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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The purpose of this research is to develop methods of generating tumor specific small peptides that will bind to human cancers. The goal is to develop an *in vivo* serial selection method of tumor-binding peptides from tumor nodules following intravenous administration of peptide phage to tumor-bearing mice. In particular, following IV infusion of naïve phage library, phage collected from a surgically harvested tumor were amplified and readministered to the same tumor-bearing animal. This process was repeated twice so that one naïve and two enriched phage samples were administered. It was identified that phage could be successfully recovered from a tumor nodule, amplified and readministered to the same organism. The mice tolerated the serial panning procedure with relatively little toxicity. Phage recovered from the final harvested tumor (3^{rd} pan) were sequenced and amino acid consensus sequences were identified. One of the sequences shared a motif with a reported MMP binding peptide.

Based on this work, the Food and Drug Administration has approved a phase I human study utilizing the protocol of the preclinical murine model system. In addition, a clinical study, based on this preclinical work, has been funded by the National Cancer Institute and is now open for accrual.

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Table of Contents

ABBREVIATIONS:

RPL- random peptide library
FDA- United States Food and Drug Administration
HB- homogenization buffer
TU- transducing unit
IHC- immunohistochemistry
HRP-horse radish peroxidase
IFA-immunofluorescence assay
ϕ-phage
LAL- Limulus Amebocyte Lysate

INTRODUCTION:

This final report describes three years of research designed to investigate the safety and effectiveness of *in vivo* screening of phage-displayed random peptide libraries (RPLs). The purpose of this research was to identify small peptides that bind specifically to breast tumor targets, which can ultimately be used to develop effective cancer therapeutics with high specificity for tumor cells and low toxicity to normal cells. Potential advantages of peptides identified by phage-display RPL technology include tumor specificity, good binding affinity, improved pharmacokinetics due to their smaller size relative to antibodies, and the possibility of developing customized ligands for individual patients. We completed toxicity testing of a variety of intravenous delivery protocols of RPLs to mice and found little toxicity in mice. In addition, serial panning experiments in tumor-bearing mice demonstrated that enrichment of phage occurs and interesting consensus sequences have been identified. Based on this data, the FDA has approved our protocol for use in humans. Approval by the University of Vermont Committee on Human Research in the Medical Sciences (CHRMS) and by the Surgeon General's Human Subjects Research Review Board (HSRRB) was also completed during the grant period. We have initiated recruitment of breast cancer patients. Three patients were evaluated for entry to the protocol during the grant period. Importantly, this work has been successfully funded by the National Cancer Institute to support human clinical studies with this protocol over the next two years.

BODY:

Task I. Construct a large panel of random peptide libraries, each with a disulfide-constrained loop and ranging in size from 8-12 amino acids.

Over the course of this research three pIII and four pVIII random peptide libraries were constructed. The third pIII library ($CX_{12}CG_4SG_3A_2$) was lengthened to 12 amino acids to increase the number of possible peptide combinations thereby improving the odds of finding high affinity peptides during the selection process. Additionally, this library incorporated a linker to provide more flexibility to the expressed peptides and to further distance the cyclic portions of the peptides from the pIII protein, possibly improving target binding.

Libraries constructed by our laboratory during the research period.

Library type	Peptide structure
pIII	$X_4CX_{10}CX_4$
pIII	$X_4\ C\ X_{11}\ C\ X_4$
pIII	$CX_{12}CG_4SG_3A_2$
pVIII	$X_4CX_{12}CX_4$
pVIII	$X_4CX_9CX_4$
pVIII	X_3CX_9CX
pVIII	$X_4CX_8CX_4$

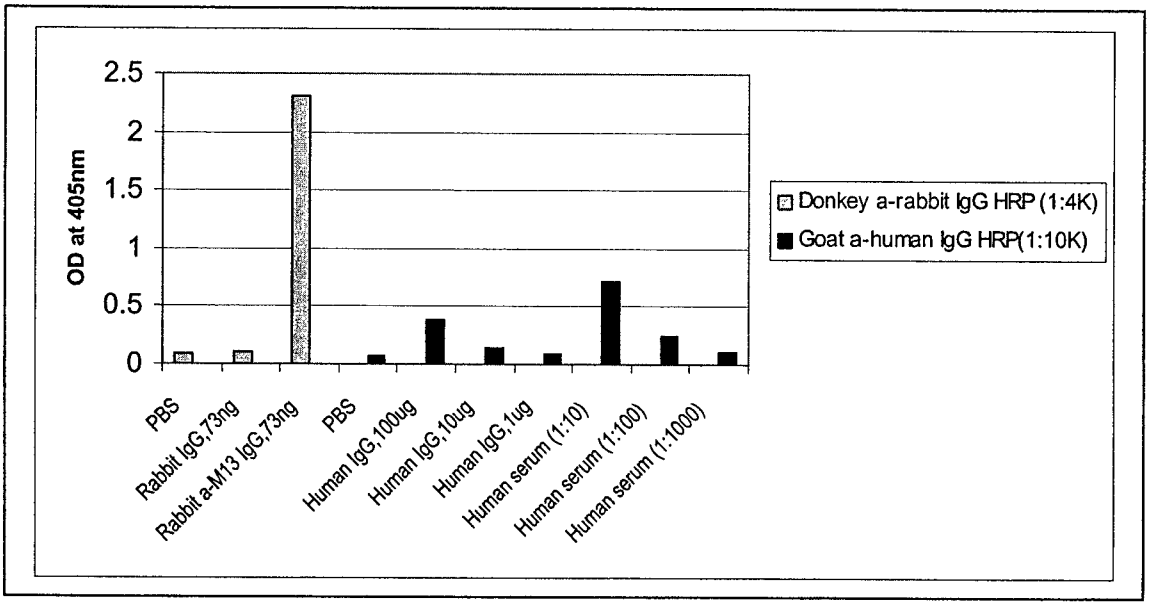
Task II. Establish the safety of intravenous administration of phage RPLs.

Pre-clinical mouse studies to evaluate the safety of phage-displayed random peptide libraries were designed and completed. This work included the development and implementation of endotoxin purification, endotoxin testing, sterility protocols, and determination of serum immune antibody response. Please refer to the manuscript in the appendix for full details of the murine toxicity study. Complete details of the data were submitted to the Food and Drug Administration to request approval for human use of an Investigational New Drug. Approval by the FDA to proceed with human clinical evaluation was obtained and was required prior to submission for approval by the Human Subject Protection Committee of the Surgeon General and of the University of Vermont. By the conclusion of the grant period all preclinical in vivo toxicity studies were completed and approval to proceed with phase I human studies were obtained by the Food and Drug

Administration (BB-IND#9145), Human Subjects Research Review Board (HSRRB) of the Surgeon General's office, the University of Vermont Committee on Human Research in the Medical Sciences, the University of Vermont Comprehensive Cancer Center Protocol Review Committee, and the University of Vermont General Clinical Research Center Advisory Committee panning.

Extensive protocol development and task assignments have been completed. An extensive protocol flow sheet of timed patient-related activities was developed (see appendix). Mock patient entry "dry runs" based on this protocol were performed to insure that all resources were present and appropriately assigned. This has been an important step since the protocol is complex. The protocol depends upon successful and timely amplification of phage. In addition all materials must be prepared in a manner with considerable attention to detail since the final product will be administered intravenously to humans. All procedures must be documented and all reagents, including otherwise minor solvents, must be accounted for and considered safe for human use. By the conclusion of the grant period we were in the process of recruiting patients for enrollment. Three potential patients were evaluated for the study but either did not meet eligibility criteria or declined to participate.

In preparation for our clinical study, an ELISA was established to evaluate human anti-bacteriophage IgG levels. This was done to determine whether a patient has pre-existing anti-phage antibodies prior to phage administration and to document the timing of development of an anti-phage antibody response. Since it is unlikely that patients have pre-existing anti-phage antibodies we do not yet have a human positive control. We have demonstrated an immune response in mice following IV administration of peptide phage. Demonstration of an immune response in mice helped establish the protocol in our lab for detection of an IgG anti-bacteriophage immune response in humans. Over the course of several assays, we determined the correct dilution for the antibodies, goat anti-human IgG HRP and donkey anti-rabbit IgG HRP. Using the appropriate antibody dilutions, another ELISA was performed using human serum from a normal volunteer (without prior known exposure to bacteriophage) as a negative control. We determined that human serum diluted 1:1000 gave a response equivalent to PBS. Additionally, we demonstrated that the phage library applied to the ELISA plate as a target was detectable by Rabbit anti-M13 IgG after incubation with donkey anti-rabbit IgG HRP, a control we routinely employ to verify that our phage have bound to the microplate.



Graph 1. Human IgG determined by ELISA, using 1 x 10^7 TU PIII library phage as target, 60 minutes post ABTS.

Task III. Identify specific tumor-binding phage by *in vivo* screening and characterize clones.

Tumor bearing MRL/MpJ-fas_{LPR} mice were serially screened three times with our peptide phage libraries. MRL mice were used for this study because they develop massive lymph node enlargement, or lymphoproliferative disease, beginning around 8 weeks of age. The lymph nodes were readily accessible for surgical harvesting. Three sequential IV injections were administered to the same mouse: (1) naïve library, (2) phage amplified from a tumor excised from the same animal (φAmp1x) after injection of naïve peptide-phage library, and (3)phage amplified from a second tumor (φAmp2x) excised from the same animal after injection of φAmp1x. Each injection occurred on separate days, in the order listed, and followed 10 minutes later by excision of tumor. Peptide-phage clones were randomly selected from the final tumor harvested and the DNA coding for the peptide was sequenced. Analysis of the peptides from the different clones revealed a number of consensus motifs. (Please see manuscript in the appendix for the description of the sequences).

Of particular interest, one consensus pattern, and one peptide in particular, had strong homology with a peptide previously shown to bind to and inhibit matrixmetalloproteinases (MMPs) 2 and 9, molecules that are strongly associated with the metastatic phenotype and are promising tumor targets[1]. In this report by

Koivunen, a peptide motif of HWGF was identified by panning against purified MMPs which bound with reasonable affinity to MMPs. Our *in vivo* mouse tumor panning experiments revealed a nearly identical motif, HWGI. We have been interested in pursuing this peptide motif to better understand possible ligands in a mouse tumor. Further investigation has also been important because it may serve as a positive control that will aid in development of methods to analyze other peptide ligands obtained from *in vivo* panning.

Immunohistochemistry evaluation of our clones (IV092499-09=CYHMVSLENGC and IV092499-20=CVLSDYIGGSC) on histologic slides of snap frozen mouse tumor did not reveal obvious binding when compared to slides of mouse brain tissue.

We then constructed peptide-phage clones to express as a fusion protein the sequences (MMP2 binder=CTTHWGFTLC and MMP9 binder=CRRHWGFEFC) that were reported by Koivunen to bind to MMP. The purpose was to obtain a positive control and to compare our clones which have similar sequences.

Preparation of MMP fusion phage: Oligos, prepared by BioSynthesis, were annealed and inserted into purified fuseV vector that had been cut with sfi1. The ligated products were then transformed into MC1061F' electrocompetent cells and grown overnight on Luria-Bertani (LB) plates supplemented with tetracycline (TET). Isolated colonies from each clone were subsequently grown in liquid culture, the dsDNA isolated using Qiagen kits and submitted with the fuse V sequencing primer (CCCTCATAGTTAGCGTAACG) to our DNA sequencing facility for confirmation of the correct sequences.

Perform MMP ELISA #1(11.16.00): MMP9 (5μg/ml) was prepared in 50mM Tris pH 7.5, 10mM $CaCl_2$, 150mM NaCl (TCN). On day 1, 500ng of target material (MMP9 or BSA) was added to the wells of a 96-well microtiter plate (Nunc MaxiSorp) and incubated overnight at 4°C. On day 2, the wells were blocked for 2 hours with 1% (w/v) casein in TBS, pH 7.4 (Pierce, Rockford IL). MMP 2-binding or MMP 9-binding phage, blocked for 30 minutes just prior to use with an equal volume of casein, were added (1×10^8 TU/well) and incubated for 2 hours at room temperature. The plate was washed 5x with 10mM Tris, 0.15M NaCl, pH 7.5 containing 0.1% (v/v) Tween 20 (TTBS), followed by sheep anti-M13 HRP (Amersham) diluted 1:1000 in PBS for 2 hours at ambient temperature. The plate was washed 5x with TTBS, ABTS (Sigma) added according to the manufacturer, and the colorimetric reaction read on a microplate reader (Bio-Tek) at 10, 30, and 60 minutes.

Initial ELISA data demonstrated that MMP2 fusion phage clone bound to MMP9 2.2x higher than BSA control and the MMP9 peptide-phage clone bound to MMP9 2.6x higher than BSA control. This level of binding was lower than that expected and was not enough for either clone to be useful as a positive control.

A variety of variables have been evaluated to increase the apparent binding of fusion phage clones MMP2 and MMP9 to purified MMP9 by ELISA. This included 1) increasing the number of phage/well, 2) using purified MMP2 (in addition to MMP9) as target, 3) assay with fresh phage instead of after titering, 4) adding negative control clones, 5) adding one of our MMP-similar clones (Clone - IV092499-16; sequence- CTGHWGIGENC) and 6) negative control target (BSA).

There was no apparent binding to MMP2, which appears due to MMP2 not binding to the plate. Binding to MMP9 seemed to be equivalent in all experiments including the negative controls.

These experiments were repeated with MMP9, Grb2, and BSA (500ng each) as targets. Grb2 is a signal transduction molecule that binds to phosphorylated residues on the intracellular domain of ErbB family. Clone MMP9 was evaluated as the expected positive binding ligand and Grb2 clone as a negative control. As an additional control to the experiment, binding to purified Grb2 target by G1 (positive control) and the putative MMP9 binding clone (negative control) was performed. G1 is a peptide-phage clone that we previously developed that binds to the SH2 domain of Grb2.

Unfortunately, MMP9 clone, library, and clone IV092499-16 all bound similarly to MMP9. G1 bound to Grb2 as expected and showed good positive control to purified Grb2.

Further attempts to develop positive controls were performed and were based on a number of reported organ binding peptide sequences which were identified by *in vivo* peptide-phage panning in a mouse model [2]. We have constructed fusion phage based on these reported organ seeking peptide sequences [2-4]. The following clones were prepared:

THP=tumor homing peptide=CNGRCVSGCAGRC
BHP=brain homing peptide=CGRECVRQCPERC
LHP=lung homing peptide=CLSSRLDAC

In the first experiment employing these clones, the tumor-homing peptide-phage clone (THP) was injected into an MRL tumor-bearing mouse. After 10 minutes, the heart was perfused with 20cc's Hanks Balanced Salt Solution (HBSS) until the liver blanched. The tumor, brain, lung, and kidney were harvested for IHC and titering. Tumor titers (TU's/mg tissue) were 6.5x and 1.6x higher than brain and lung respectively. Kidney titers however were 1.9x higher than tumor. No phage were seen by anti-M13 IHC (at same dilution that showed staining in toxicity studies).

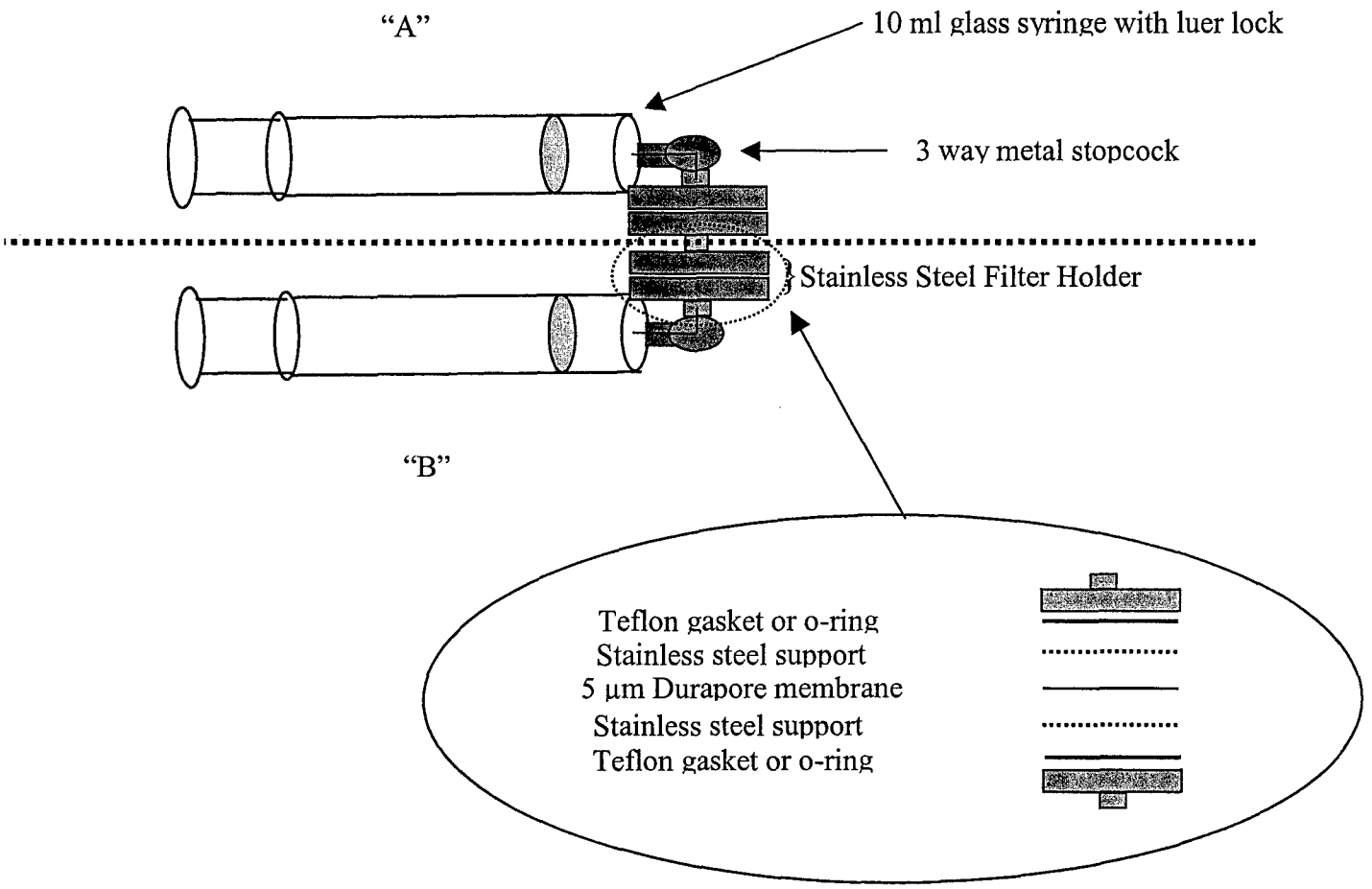
In a subsequent experiment, brain-homing peptide-phage (BHP), lung-homing peptide-phage (LHP) and naïve library (Lib) were injected IV (1 x 10^8TU's) into each of three BalbC mice. As before, the phage was allowed to circulate 10 minutes, the heart perfused, and organs harvested. Brain, lung, and kidney were then subjected to IHC and titering analyses. Tissue titers for BHP showed 22-fold and 5.6-fold more phage in lung and kidney than in brain. LHP tissue titers showed lung to be 48x and 3.2x higher than brain and kidney respectively. However lung titers from the naïve library injected mouse showed greater amounts of infective phage (1.4x) compared to the LHP-injected mouse. IHC results on these tissues were negative for staining. Based on this data we were unable to confirm the results that reported that these phage-displayed peptides homed to brain, kidney, or lungs.

An additional issue was to insure that we had low background from unbound phage in the blood during phage collection from the mouse. This required drainage of the vascular system of the mouse and perfusion with saline. We confirmed that the method of mouse whole body perfusion methods were satisfactory using blue dye. We observed that superficial structures, extremities (tail, paws, nose) turned blue. The heart, kidney, tumor, and liver were all blue upon dissection. Blue staining of the lung and spleen were less conclusive. When the lungs deflated it was difficult to discern interior vs. exterior when cut in half. The chest cavity fills with dye from spillage of the perfusate (from the catheter inserted into the heart) and stains tissues in the chest cavity. Brain tissue was not blue, but vessels in base of skull (carotids) appeared blue. Repeat experimentation with more attention to chest cavity spillage demonstrated that vessels in the lung were in fact blue stained as were blood vessels in the brain.

In vivo panning has the advantage of a multiplicity of targets for possible binding by the phage displayed RPL. This asset is also a problem downstream in terms of target identification and evaluation of ligand-target interactions. The method most readily available to us for determining the approximate affinity and binding location of the ligand was to incubate candidate peptide-phage clones directly on a histologic tissue slice prepared from the mouse tumor. Anti-M13 phage antibodies were incubated on the slide to detect bound phage. We have learned that this technique is not straightforward and may not be an optimal method to accomplish the goal of determining whether a candidate clone has affinity for the tumor or the approximate location of the target. There are several variables that are difficult to control, for example, determination of the optimal concentration of phage to place on the histologic specimen. As with conventional IHC with antibodies, each antibody must be evaluated for the appropriate dilution to its cognate target. Even with antibodies known to bind to specific targets, an incorrect concentration will result in either false negative or false positive results. Determining appropriate peptide-phage clone concentration for IHC, particularly with unknown binding properties, is challenging. Another significant problem is that the target of the ligand may be present on the histologic slide in very small quantities. In this case, even a phage with good binding affinity may not yield a detectable signal. We believe that these problems hampered the ability to characterize clone affinity and binding characteristics.

We have sought additional methods to select and characterize clones. One of the potential problems is that peptides identified from panning experiments commonly have low affinity for their target. One possible solution is to extend the panning series to select for clones with higher affinity. This would not be feasible in the human clinical situation. A possible alternative would be to extend tumor panning sessions using an *ex vivo* model following an initial in vivo pan. A system has been devised and preliminarily evaluated for panning fresh tumor tissue ex vivo. The goal was to simulate as much as possible the presentation of targets available with in vivo panning and to provide a method of negative subtraction of phage that bind to normal tissue. This approach could provide a possible method to further select and possibly characterize ligands harvested from a patient's tumor.

An apparatus was designed to allow positive selection of phage to a freshly excised tumor and negative selection to freshly excised normal tissue. Two containment vessels were devised such that freshly excised tumor could be placed in one vessel and normal tissue in the other. The two vessels were connected with tubing with in line filters. The choice of filter size allowed the phage library to move freely back and forth between the tumor and normal tissue compartments while preventing tumor or normal tissue from leaving its respective compartment.



A simple apparatus was set up using two glass syringes as the containment vessels for tumor and normal tissue (see diagram). Preliminary evaluation of the system

was performed. The syringe set-up illustrated above was pre-blocked with 4% normal human serum for at least 30 minutes prior to use. A small amount of normal breast tissue (304 mg) was finely minced and placed in a small volume of 4% (v/v) normal human serum, placed in syringe A and allowed to mix gently on a nutator. Library phage (1.12×10^{10}TU) was blocked with 4% serum at ambient temperature for approximately 1 hour and then added to the normal breast cells in syringe A and allowed to nutate for 10 minutes. The peptide-phage solution was then passed through the membranes and into syringe B containing 1×10^6 SkBr3 tumor cells in 4% human serum, trapping the normal cells on membrane A. The peptide-phage were allowed to incubate with the tumor cells for 10 minutes. This process was repeated twice more. Following the third 10-minute incubation of phage with tumor cells, the phage solution was filtered to a waste container, trapping the tumor cells on membrane B. The membrane and syringe were rinsed with 15 x 10 ml phosphate buffered saline (PBS) to remove unbound peptide-phage. Rinses were subsequently cytospun, stained and viewed microscopically to check for cell loss.

Stainless steel filter holder B was disassembled and the stainless steel support (positioned between the membrane and the syringe) and the membrane were treated with 100mM triethylamine (TEA) for 10 minutes to disrupt the cell membranes to expose any internalized peptides. The TEA solution was then treated with 0.1 volume of 1 M Tris HCl, pH7.4. The membrane was further treated with pH 2.3 and pH 12 buffers to elute any remaining bound phage. All eluates from these treatments were used to infect K91 *E.coli* grown to mid-log phase. This culture incubated at 37°C at 100rpm for 1 hr. Tetracycline was added (0.2μg/ml) for selection and the culture incubated at 260rpm an additional 25 minutes and incubated on ice for 10 minutes. Aliquots were plated on Luria Bertani (LB) tetracycline-supplemented plates and incubated overnight at 37°C. Resulting clones from this experiment were subsequently grown in culture and stored at –80°C in 5% DMSO.

Results.

- No cells were observed in the rinses collected following phage incubation with the tumor cells, indicating an intact membrane used to trap the cells following the final rinse. Phage were readily able to cross the membranes. This insures that phage could freely move back and forth between the tumor and nontumor compartments and that there was no cross contamination of cells across the compartments.
- Rinse 15 had a low titer of 15.3 TU/ml, indicating acceptable removal of unbound phage from the system. This indicated that the apparatus did not

retain significant quantities of phage that would otherwise increase the background level of nonspecific phage.

- 3.85 x 10^7 TU, representing0.34% of the phage used, were recovered from the membrane and support following a single pan. This represents the fraction of phage presumably bound to cancer cells trapped on the membrane and support after clearance of unbound phage. This indicates that this apparatus was effective in allowing the majority of unbound library to be rinsed away from the target tissue.
- Further panning experiments using serial panning to SkBr3 cells yielded clones with affinity for the target cells (see IFA results Graph 2 below).

In order to develop an alternative method to screen clones for tumor binding affinity, an immunofluoresence assay (IFA) was developed using a 96 well plate format. This would potentially enable us to screen many clones in a single assay. It does however require cells to adhere as a monolayer. This means that tumor cells would need to be disaggregated from the primary tumor. To the extent that this would alter targets, the value of this as a screening tool lessens.

The preliminary evaluation of this 96 well method was performed with SkBr3 cancer cells.

Clone preparation. Each clone to be screened for binding to SkBr3 cells was grown overnight at 37°C in 40 ml of LB supplemented with tetracycline. Cultures were then spun at 8000rpm, at 4°C for 10 minutes to pellet the bacteria. The supernatant was passed through a 0.2μm PES membrane to remove any residual bacteria and 0.15 volumes of PEG-NaCl were added to precipitate the peptide phage. After a 30-minute incubation on ice, the solution was centrifuged for 20 minutes at 9000rpm and the resulting peptide-phage pellet was resuspended in 200μl PBS and stored at 4°C until use. Clones were prepared up to a week ahead as equipment and labor considerations limited us to preparing 20 clones at any one time.

IFA Clone Screening Protocol. SkBr3 cells in medium supplemented with gentamicin (20μg/ml) were plated (20,000/well) in a 96-well black, clear-bottom polystyrene plate (Costar #3603). Cells were incubated for ~72 hrs at 37°C, 5%CO2 to adhere and form a monolayer.

On the day of assay, the medium was removed and the cells were fixed with pre warmed 2%(v/v) paraformaldehyde (PFA) for 30 minutes at ambient temperature. Cells were rinsed twice with PBS and then blocked for 30 minutes at ambient temperature with 10%(v/v) normal goat serum. The plate was then blocked for 1 hr with Casein Blocker (Pierce). Clones were blocked with an equal volume of 20%normal goat serum in casein for 30 minutes, at room temperature

while mixing on a nutator. The pre-blocked clones were then added to the plate to all but the control wells, and incubated for 2 hrs.

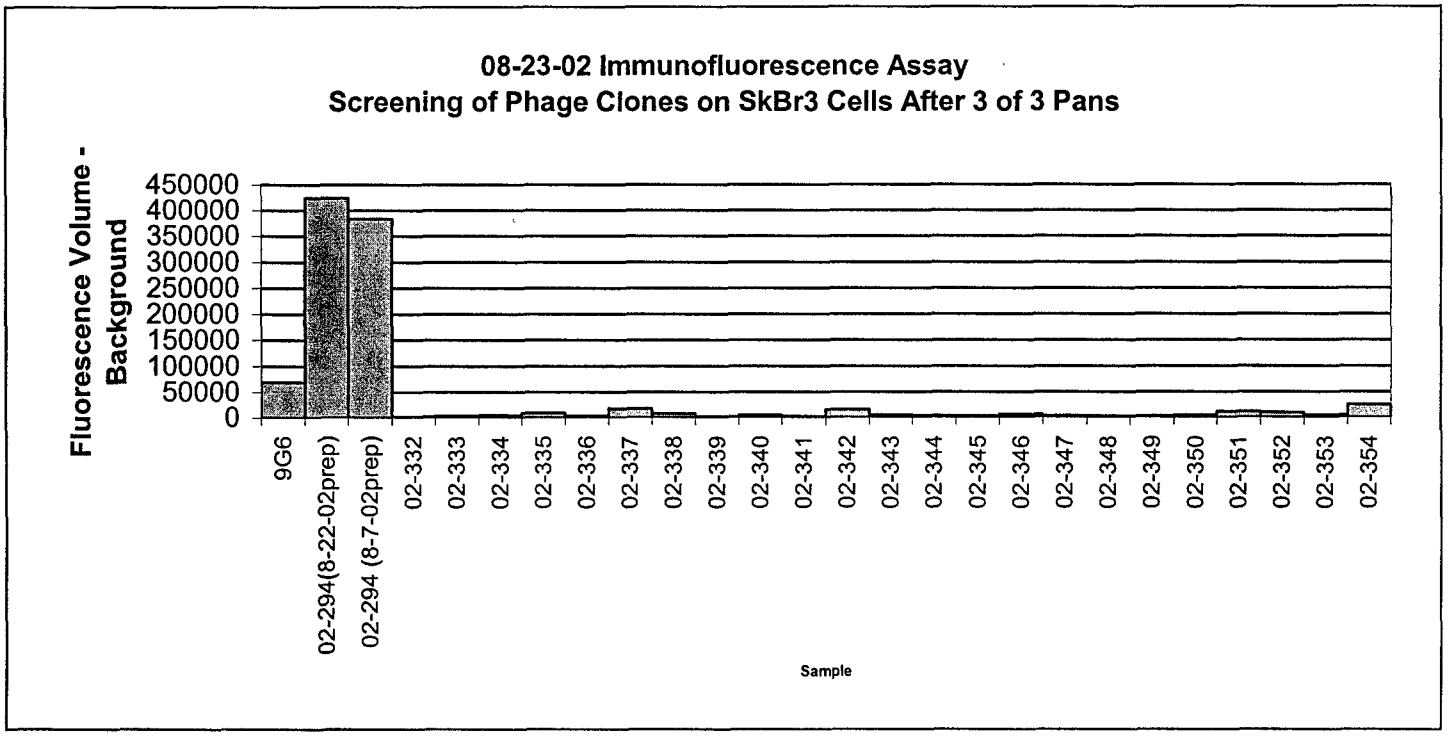
The plate was washed 5x with 0.1% (v/v) Tween in PBS (TPBS). To the negative control well, TPBS was added, and 9G6 diluted in TPBS (SantaCruz SC-08) was added to the positive control well. Mouse anti-M13 (Amersham #27942001) was added to the phage-treated wells. The plate was allowed to incubate for 2 hrs, washed 5x with TPBS and Goat anti-mouse Alexa Fluor 488 conjuate (Molecular Probes A-11001) was added to all wells and allowed to incubate for 1 hr. The plate was then washed 5x with TPBS, 100µl TPBS added to each well, and imaged using the Molecular FX Imager in the Vermont Cancer Center Core Facility at the University of Vermont.

Approximately 100 clones were selected from a single panning event of the SkBr3 cells in the syringe panning apparatus. These clones were evaluated in the 96- well format for binding as described above and no strong binders were found.

Additional panning was performed with the syringe panning apparatus. Instead of a single pan, three serial pans were performed and negative selection of phage was not used. Phage recovered from each pan were amplified and used for the next round of panning.

Approximately 100 clones from the 3rd of 3 Serial Screenings were assessed by IFA in the 96 well format as described above. Of the approximately 100 clones assayed for binding on SkBr3 tumor cells, one clone (02-294) stood out from the rest, far surpassing even the positive control. A second preparation of this clone was cultured and assayed again and compared to the first. The results are shown below.

Graph 2.



This method appears promising and further evaluation of this 96 well phage binding assay will be to use cells from the patient tumor as the target instead of cell-line cells. It is hoped that the combination of both ex vivo panning and clone evaluation against whole tumor cells will facilitate and complement in vivo panning.

Task IV. Synthesize and characterize free peptides which bind to breast-cancer specific targets

We have assessed a variety of methods for determining binding affinity of peptides. As positive control we used a peptide against ErbB2 that we generated in our lab by panning against purified ErbB2. We evaluated commercial affinity measuring devices, for example calorimetry. The devices available within our budget did not meet expectations. We have synthesized a biotinylated version of the ErbB2 binding peptide and generated a number of affinity curves. The binding experiments were performed by incubating ErbB2-binding biotinylated peptides across a range of concentrations with purified extracellular domain of ErbB2. The sensitivity of the assay appears low even after a 24 hour incubation of peptide and target. We are currently synthesizing a fluorescently labeled peptide in order to improve sensitivity of the assay. If the fluorescently labeled peptide provides insufficient sensitivity, we will synthesize radiolabeled peptides for binding affinity experiments.

Synthesizing peptides is expensive and time consuming. We are also attempting to optimize a technique of determining relative binding affinities of peptide-phage clones by incubation with target for 24 hours across a range of phage particle concentrations. This would facilitate evaluation of peptide ligands without having to synthesize peptides for each candidate ligand. Although free-peptide Kd's would not be established in this manner, it would provide important information on the relative binding affinity of different candidate clones.

At the conclusion of this grant we did not reach the point of identifying a tumor binding phage clone that had sufficient binding affinity to warrant synthesis and characterization of the free peptide. The major goal of the study was to evaluate and develop the *in vivo* phage selection system. Task 4, although incomplete was a logical next step for evaluation of phage clones that demonstrated good tumor binding.

One clear advantage of *in vivo* panning is that the target need not be known prior to panning. This in fact may be one of the major advantages of this system

since there are likely many valuable targets that are today unknown. However, it has become clear that once a clone has been selected, there is considerable advantage and maybe even a necessity to identify the target cognate to the clone. It appears that in order to determine binding affinity the target must be known. This will allow initial characterization of selected clones. In addition, it is common for peptides displayed on phage to have only modest binding affinity. The steps involved with modification of the peptide to obtain higher affinity are reasonably straightforward, for example using a biased library based on a limited motif of the initial clone. However, selection of clones from this biased library and evaluation of binding affinity requires an ample amount of target.

We are currently addressing the important problem of identifying the targets of clones obtained by *in vivo* panning. This exceeds the scope (and time) of this current funded research.

KEY RESEARCH ACCOMPLISHMENTS:

- Construction of 7 random peptide libraries with a variety of ring sizes and linear side arms.

Library type	Peptide structure
pIII	$X_4CX_{10}CX_4$
pIII	$X_4\ C\ X_{11}\ C\ X_4$
pIII	$CX_{12}CG_4SG_3A_2$
pVIII	$X_4CX_{12}CX_4$
pVIII	$X_4CX_9CX_4$
pVIII	X_3CX_9CX
pVIII	$X_4CX_8CX_4$

- Design of comprehensive pre clinical studies to evaluate the safety of *in vivo* screening with phage-display random peptide libraries in an animal model
- Completion of pre clinical animal studies designed to evaluate and predict the safety of performing *in vivo* screening with phage-display random peptide libraries in human cancer patients. The preclinical studies demonstrated that repeat administration of phage displayed random peptide library in the same animal did not have notable serious sequelae.
- Consensus sequences were recovered from mouse tumors following serial panning *in vivo*.
- Further analysis of some of the peptides identified from peptide phage collected from mouse tumor tissue following serial *in vivo* panning have

high homology with peptides known to bind to and inhibit MMP2 and MMP9.

- Completion and submission of an Investigational New Drug application to the FDA
- Response to revisions following FDA review.
- Issuance of an Investigational New Drug approval by the Food and Drug Administration to perform human studies of administration of phage-displayed RPL and tumor panning (BB-IND#9145)
- Construction of mouse brain-homing, lung-homing, and tumor-homing phage clones based on reported peptide sequences. *In vivo* assays in mice were performed with the expectation that these clones would provide positive controls. However, homing of these clones to their respective organ was not observed. .
    - Tumor-homing peptide (CNGRCVSGCAGRC)
    - Lung-homing peptide (CGFECVRQCPERC)
    - Brain-homing peptide (CLSSRLDAC)
- Demonstration of immune response in mice following administration of phage-displayed RPL
- Approval by the University of Vermont Committee on Human Research in the Medical Sciences for the human cancer patient study of administration of phage-displayed RPL and tumor panning
- Approval by the University of Vermont Comprehensive Cancer Center Protocol Review Committee for human cancer patient protocol to study administration of phage-displayed RPL and tumor panning.
- Approval by University of Vermont General Clinical Research Center Advisory Committee for human cancer patient protocol to study administration of phage-displayed RPL and tumor panning
- Approval for support by University of Vermont General Clinical Research Center. Extensive protocol development and task assignments have been completed. Mock patient entry "dry run" performed to insure that all resources present and appropriately assigned.
- Submission of Patent on *In Vivo* method of phage display to the United States Patent Office.
- Initial development of human IgG immune response assay.
- Development of tumor containment system for in vitro selection of peptide phage on human breast tumor and library subtraction on normal breast tissue
- Development of immunofluoresence assay (IFA) to screen for peptide phage that bind to SkBr3 tumor cells
- Discovery of a clone that binds to SkBr3 cells as assessed by IFA

- Obtained successful funding from the National Cancer Institute to continue this work in a human clinical trial of *in vivo* panning with phage-displayed random peptide library.
- Publication of manuscript by Cancer Chemotherapy and Pharmacology "Phage-displayed random peptide libraries in mice: toxicity after serial panning title" (in press, available on-line, attached in appendix to this report)

REPORTABLE OUTCOMES:

- Investigational New Drug approval by the Food and Drug Administration (BB-IND#9145).
- The activities of this grant were submitted as part of the University of Vermont Comprehensive Cancer Center Grant Application. Our section was scored high and the Cancer Center Grant has subsequently been approved for five years of funding.
- A manuscript detailing the mouse toxicity data and the initial results of peptide ligands obtained from the harvested tumors has been accepted and is available on-line pending written publication.
    - Krag DN, Fuller SP, Oligino L, Pero SC, Weaver DL, Soden AL, Hebert C, Mills S, Liu C, Peterson D (2002) Phage-displayed random peptide libraries in mice: toxicity after serial panning. Cancer Chem and Pharm DOI 10.1007/s00280-002-0489-4, *in press*
- An invited presentation of this research data will presented to the Northern New England Clinical Oncology Society on Oct 26, 2002 presentation is planned at
- A patent application has been submitted: *In Vivo* Methods for the Identification of Target Specific Binding Molecules in a Human and Their Use in Cancer Detection
- New funding was awarded to us based on this research: NIH PA-00-047 Quick Trials for Novel Cancer Therapies, Project title: *In Vivo* Selection of Ligands for Targeted Therapy

CONCLUSIONS:

Serial administration of a phage-displayed random peptide library is relatively nontoxic in a murine model. This was performed in a scheme preparatory to human clinical studies. Therefore this model has direct relevance to cancer patients. This research demonstrated that animals would tolerate a series of intravenous administrations of a peptide-phage library, tumor harvesting, and readministration of phage amplified from the harvested tumor. The initial phage

administration was naïve library. Subsequent administrations of phage were the amplified phage collected from the tumor.

Two possible side effects were addressed by this series of studies. The first was the possible reaction to naïve phage library. In this situation the biggest concern was related to the phage and not to the peptide displayed on the phage. This is because the copy number of each peptide was low in the range of 10 to 1000 molecules. Even a very toxic peptide should have little to no toxicity at that concentration. The phage collected from the tumor were then amplified which considerably increased the copy number of the displayed peptides. The mice tolerated both formulations.

This research also demonstrated that phage could be successfully harvested from a lymphoid tumor in sufficient quantities that, following amplification, a repeat administration could be performed. We also conclude that consensus sequences could be identified from harvested clones. The identification of consensus sequences indicated but does not confirm that clones harvested from tumors were being enriched due to tumor binding. The identification of a consensus motif that was very similar to peptides reported to bind to MMPs further indicates that tumor binding peptides were recovered from the surgically harvested mouse tumor.

It is our conclusion that there is sufficient rationale and safety to continue with a phase I clinical study. Concurrence of this conclusion is supported by the FDA (issuance of IND) and the National Cancer Institute (award of funding).

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APPENDICES:

Manuscript

Flowcharts:

- Research Laboratory Flowchart for Peptide Phage
- Patient Flow Chart
- Tissue Procurement Flow Chart

Cancer Chemotherapy and Pharmacology

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Original Article

Phage-displayed random peptide libraries in mice: toxicity after serial panning

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Abstract

Purpose. In vivo screening of phage-displayed random peptide libraries (RPLs) has been used to identify peptide ligands to targets found on endothelial cells of blood vessels supplying specific tissues such as brain, kidney, and tumor tissue. Peptides that bind specifically to blood vessels supplying tumor tissue have been conjugated to cytotoxic agents and used to successfully eradicate tumors in a mouse model. With the ultimate goal of developing similar methods for treating human cancer, we describe an in vivo RPL screening process that, unlike previous in vivo experiments, does not harm the animal being screened.

Methods. RPLs were administered to FVB, BalbC, and tumor-bearing MRL/MpJ-fas$_{LPR}$ mice in a variety of dosing formats. Tumor nodules were excised 10 min following infusion and phage were amplified from the specimens. Phage were reinjected into the same animal within 48 h. This process was repeated twice for a total of three in vivo screens of mouse tumor tissue within the same animal. Mice were observed for systemic side effects, histopathologic damage, and presence of phage in organs. Peptide sequences were determined from several third-pan phage clones.

Results. Overall there was minimal toxicity from administration of single or repeat doses of RPLs. Amino acid consensus sequences were identified and some of the sequences were similar to those of peptide ligands that bind matrix metalloproteinases.

Conclusions. Serial administration of an RPL is well tolerated and serial panning in individual mice leading to consensus sequence motifs is possible. Based on these preclinical data the Food and Drug Administration has approved the implementation of human clinical trials with this technique.

Keywords. Phage-displayed random peptides - Toxicity - Ligands - Cancer - Mouse

Abbreviations. *IHC:* immunohistochemistry *PBS-EPI:* phosphate-buffered saline and eukaryotic protease inhibitors *PBS-PPI:* phosphate-buffered saline and prokaryotic protease inhibitors *RPL:* phage-displayed random peptide library

TU: transducing unit

Introduction

Approval of trastuzumab (Herceptin; Genentech, South San Francisco, Calif.) in September 1998 by the Food and Drug Administration for the treatment of breast cancer was a major clinical milestone in the field of targeted therapeutics [4, 51]. Despite the recent and exciting success of Herceptin and other notable exceptions [23, 28, 49], the field of antibody-based therapy and diagnosis of solid tumors is extremely limited. Antibody-based therapy has a number of important limitations that have prevented its rapid development and translation to the clinic. Failure of antibodies in the clinic is likely due to unfavorable pharmacokinetics, lack of tumor penetration, immunogenicity, and undesirable uptake by the reticuloendothelial system [9, 12, 15, 17, 18, 19, 22, 30, 42]. Should the antibody be conjugated to a toxic molecule, destruction of normal cells in the reticuloendothelial system may limit the deliverable dose [40]. In addition, antibodies are complex biologic molecules that are not readily prepared for human use. Efforts to diminish the size of the antibody molecule, for example single-chain antibodies of about 25 kDa, have resulted in improved pharmacokinetics. There are clear indications that, for ligands, smaller is better [24, 27, 38, 45, 58, 62, 63].

Peptide ligands (1 to 2 kDa) that are much smaller than antibodies (150 kDa) but still retain selective binding affinity to target molecules may overcome several of the limitations of antibody therapy. An attractive source of peptide ligands is phage display technology [32, 44, 48]. Phage-displayed random peptide libraries (RPLs), which contain 10^6 to 10^8 different peptide sequences, are particularly powerful in that the peptide ligands are physically linked to their encoding DNA. DNA is easily amplified for sequencing and the sequence of one binding peptide out of millions of non-binding peptides can be determined. Although a variety of formats have been developed, the most common phage-displayed RPL involves insertion of random synthetic DNA into the gene coding for the minor coat protein pIII [47] or the major coat protein pVIII [20, 29]. The foreign DNA is expressed as random peptides in limited copies at the free N-terminus of pIII or pVIII. Each phage particle displays a different peptide.

Phage-displayed RPLs have been used to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of clinically important targets including cell membrane receptors [3, 14, 46, 54, 59, 60], tumor-associated antigens [5, 11, 53], hormones and cellular messengers [6, 26], matrix-related elements [21, 31, 57], immunoglobulins [10], DNA [7], intracellular signal transduction molecules [13, 35], and nuclear receptors [33].

Most of these ligands have been identified using in vitro screening techniques which involve binding the target (purified protein or cells) to a matrix, incubating immobilized target with the peptide-phage library, washing away nonspecific binders, eluting specifically bound phage, amplifying eluted phage, and sequencing the DNA to determine the identity of the peptide responsible for the binding activity. In vivo RPL screening in animal models has resulted in ligands to organ-specific vasculature and to implanted tumor xenografts [2, 36, 37, 41, 43]. Administration to tumor-bearing mice of peptide-doxorubicin conjugates with affinity to tumor xenografts has been shown to result in a marked decrease in doxorubicin toxicity, selective tumor destruction, and improved animal survival [2]. These results are rather dramatic and represent a promising and unique approach to clinically relevant targeted therapeutics.

We are interested in adapting and applying in vivo ligand selection to human cancer patients. In preparation for human clinical studies, we evaluated in a murine model the toxicity of repeated administration of naive (full primary library) and enriched RPLs in conjunction with tumor harvesting and collection of phage. The results of this preclinical study are presented here and are the basis for approval by the United States Food and Drug Administration (Investigational New Drug BB-IND#9145) to begin human cancer patient studies.

Materials and methods

Phage-displayed RPL

The RPL used in these studies was constructed in the fUSE5 gene III phage-display system [10, 47]. The fUSE5 vector and *E. coli* host strains were a generous gift from Dr. George Smith at the University of Missouri. The half-site cloning

method used by Cwirla et al. was employed in RPL construction. The following oligonucleotides were synthesized, annealed and ligated into 10 μg of SfiI-digested fUSE5 phage vector: 5′ pGGGCTTGC(NNK)$_9$TGCGGGGCCGCTG 3′, 5′ GCAAGCCCCGT 3′, and 5′ CGGCCCCGCA 3′, where N represents a position in the oligonucleotide with an equal chance of being occupied by G, A, C or T, and K represents a position with an equal chance of being occupied by either G or T. The ligation was transformed into electrocompetent *E. coli* (MC1061F') cells using electroporation (BIO-RAD E.coli Pulser;BIO-RAD, Hercules, Calif.). Ligation of an insert into the fUSE5 tetracycline-resistant vector causes a gene III frame-shift. A vector without an insert is non-infective and therefore will not grow on a tetracycline-containing agar plate. Therefore, only clones having a frame-restoring insert can contribute infectious particles to a library and the library complexity can be determined by counting the original number of transformants. After electroporation, the library was amplified overnight to produce thousands of copies of each peptide-phage particle library member. DNA sequencing of the N-terminal region of gene III of randomly chosen phage clones confirmed the presence of correct inserts. The peptides were nine amino acids long and flanked by cysteine residues believed to form disulfide-linked cyclic peptides. While each phage displays only one specific peptide, the complexity of this library contains phage displaying approximately 2×10^6 different peptides.

Preparation of the RPL for intravenous injection

Peptide-phage were prepared from *E. coli* cultures grown at 37°C overnight on 2×YT medium agar plates (Tryptone 16 g/l and yeast extract 10 g/l; Mikrobiologie). The plates were supplemented with kanamycin (Sigma, St Louis, Mo.) and tetracycline (Sigma). fUSE 5 contains a tetracycline-resistance gene that allows phage-infected *E. coli* to grow as colonies in the presence of tetracycline. The phage particles were resuspended in phosphate-buffered saline containing prokaryotic protease inhibitors (PBS-PPI; Sigma P8465). The phage suspension was centrifuged twice to remove bacterial cells and filtered with a 0.22-μm polyethersulfone membrane (Corning, Corning, N.Y.) to completely remove any remaining *E. coli* cells. The phage were concentrated by precipitation with 0.15 ml cold polyethylene glycol per milliliter of filtrate and the precipitate centrifuged. The resulting pellet was resuspended in fresh PBS-PPI and filtered through a pyrogen-free 0.2-μm cellulose acetate filter (Schleicher & Schuell, Keene, N.H.).

Endotoxin removal and testing

Endotoxins were removed from the preparation by performing three 1% (v/v) Triton X-114 (Sigma) extractions [*1*]. The phage were concentrated with polyethylene glycol again and the resulting pellet resuspended in PBS-PPI by shaking for 10 min at 200 rpm on ice followed by centrifugation. The resulting suspension containing the peptide-phage was passed through a 0.45-μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2-μm cellulose acetate filter to sterilize the preparation. The Limulus Amebocyte Lysate gel clot assay (Endosafe, Charles River Laboratories, Charleston, S.C.) was used to determine the level of endotoxins remaining in the preparation and to check for potentially interfering substances in the preparation that might inhibit the gel clot reaction.

Sterility testing

Sterility of the phage preparations was tested according to the guidelines of the US FDA Code of Federal Regulations (21CFR610.12), by inoculation of the preparation into fluid thioglycolate medium and tryptic soy broth (Difco Laboratories, Detroit, Mich.). These tests confirmed the sterility of the preparations, as expected after 0.2-μm filtration.

Description of mice

Three strains of mice were used for the toxicity studies: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL) (Jackson Laboratories, Bar Harbor, Me.). FVB and BalbC mice are normal strains. MRL mice, beginning around 8 weeks of age, develop lymphoproliferative disease resulting in massive lymph node enlargement. MRL mice were chosen for in vivo screening because they formed multiple, large, superficial tumors that were surgically accessible. Tumors chosen for excision varied in size from approximately 5 to 10 mm. MRL mice have the disadvantage of dying rather early and

somewhat unpredictably compared to other strains of mice.

Survival surgery protocol

The University of Vermont Institutional Animal Care and Use Committee approved all animal procedures. Mice were weighed, positioned on a warming pad to maintain body temperature, and anesthetized with halothane. Ophthalmic ointment was applied to the eyes which were protected from bright light. Breathing pattern and toe pinch were used to monitor the level of anesthesia. A warm compress was applied to dilate the tail vein. Through a 29-gauge needle, 250 µl or less of sterile peptide-phage preparation was injected into the tail vein. Electric clippers were used to shave the area immediately surrounding the tumor to be excised. Isopropyl alcohol was used to cleanse the operative field and sterile drapes and instruments were used. The subcutaneous tumor of interest was excised through a small skin incision 10 min following injection. The incision was closed using interrupted 5-0 nylon sutures. Mice were injected subcutaneously with buprenorphine (0.05 mg/kg) for pain and again 12 h after surgery.

Collection of phage from harvested tumors

The tumor was rinsed with phosphate-buffered saline-eukaryotic protease inhibitors (PBS-EPI), weighed, minced and homogenized in homogenization buffer (RPMI supplemented with 1.8 µg/ml insulin, 2 m*M* L-glutamine, and 10% calf bovine serum). The homogenate was centrifuged and rinsed several times with homogenization buffer to eliminate unbound phage, and the final pellet was resuspended in homogenization buffer. An excess of Kan *E. coli* cells were added to rescue the remaining tissue-bound phage. The suspension was incubated while gently shaking for 1 h at 37°C, followed by the addition of tetracycline (0.2 µg/ml) and a 25-min incubation with vigorous shaking at 37°C. The suspension was centrifuged and the supernatant containing peptide-phage removed for quantitation and amplification. An aliquot was saved for titering. The remainder of the rescued phage were plated on 2 × YT agar plates supplemented with kanamycin/tetracycline and amplified overnight. Amplified peptide-phage (φAmp1 ×) was subsequently harvested and purified for injection as described above in Methods.

Quantitation of phage in harvested tissues

Tissues for titering were weighed, homogenized with disposable pestles in a small amount of PBS-EPI, and incubated with an equal volume of *E. coli* cells for 1 h at ambient temperature. Tetracycline (0.2 µg/ml) was added to the suspension followed by vigorous shaking for 25 min at 37°C. The suspension was concentrated by centrifugation (6600 *g*, 5 min at 4°C), resuspended in approximately 50 µl PBS-EPI and plated on LB Kan/Tet plates (Luria-Bertani). Plates were incubated overnight at 37°C. Each colony represented one transducing unit (TU). In some cases, heparinized blood (20 µl) was also titered essentially as described above, but without homogenization.

DNA and amino acid sequence determination of harvested phage

Several isolated *E. coli* colonies from the final peptide-phage amplification titer plates were grown further in 5 ml LB broth in order to amplify and isolate the DNA (Qiagen mini-prep kit; Qiagen, Valencia, Calif.). Using this DNA as a template, DNA sequence analysis was performed using the fUSE5 sequencing primer CCCTCATAGTTAGCGTAACG. The amino acid sequences of peptides displayed by peptide-phage eluted from tumor tissue were deduced from the DNA sequence of the corresponding phage clones. Consensus sequence identification was performed by visual inspection and with the PILEUP program in the GCG DNA analysis software package (Wisconsin package, Genetics Computer Group, Madison Wis.).

Analysis of organs

Mice were killed and samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen, gonads, and liver) were immediately harvested from each mouse for analysis by (1) hematoxylin & eosin staining to assess pathology, (2) immunohistochemistry (IHC) to look for the presence of phage particles, and (3) phage

titering to determine the number of infective phage. Organ pieces were fixed in buffered formalin, embedded in paraffin, sectioned and mounted onto slides. Rabbit α-M13 (Sigma) 7.3 and anti-rabbit polymer HRP (Dako) were used for IHC evaluation of phage. Positive control tissues for phage IHC were prepared from the organs of a mouse that had phage injected 10 min prior to organ harvest. The project pathologist (D.L.W.) evaluated all slides.

Systemic toxicity endpoints

Animals were observed for signs of toxicity by daily monitoring of behavior (posture, activity level, and grooming), gross appearance (coat), and body weight.

Protocols for intravenous administration of RPLs

Group-1 mice: single injection of naive phage. Eight mice (four FVB, four BalbC) were injected with a single dose of naive library phage. Two control mice (FVB) were injected with saline. Two mice of each strain and one control were killed for organ harvest at 3 days (to assess acute toxicity). The remaining mice were killed for organ harvest at 3 weeks (to assess chronic toxicity).

Group-2 mice: single injection of phage amplified from a tumor. Seven mice (three FVB, four MRL) were treated with 6.4×10^8 TU of peptide-phage that had been passaged through tumor tissue once (φAmp1 ×). A second set of eight mice (four FVB, four MRL) were injected with 8.2×10^{10} TU of peptide-phage that had been passaged through tumor tissue twice (φAmp2 ×). Two mice of each strain were killed for organ harvest at 3 days (to assess acute toxicity) or 3 weeks (to assess chronic toxicity).

Group-3 mice: serial injection phage. Six mice (MRL) were injected with 3.8×10^9 TU of naive peptide-phage. At 48 h intervals the mice were additionally injected with 3.6×10^9 TU of φAmp1 × and then 2.8×10^9 TU of φAmp2 ×. Blood was drawn 4 days following injection, and then twice a week until it was shown to be clear of infective phage by titering. The mice were killed at 3 weeks and organs harvested for analysis.

Group-4 mice: serial injection of phage and tumor harvesting. Three MRL mice each bearing at least three palpable tumors were evaluated. Naive RPL was injected and 10 min later a tumor nodule was excised. The animals were allowed to recover. Phage were recovered from the tumor, amplified and labeled as φAmp1 ×. The mice were injected 1 to 2 days later with φAmp1 × recovered from their own tumor. After 10 min a second tumor was excised and the mice were again allowed to recover. Phage recovered from tumor 2 was labeled φAmp2 ×. The mice were injected 1 to 2 days later with φAmp2 × recovered from their own tumor. A third tumor was excised 10 min after intravenous injection of φAmp2 ×, the incision sutured and the animal allowed to recover. Peptide-phage were eluted from the third tumor and amplified for DNA sequence analysis. Mice were killed 3 weeks following the third phage injection/surgery and organs were harvested for analysis.

Immune response by ELISA

Over 3-5 days, BalbC mice were injected two or three times with phage library (9.4×10^8 TU/dose). Blood was drawn from the saphenous vein for serum IgG measurements prior to phage administration and at 1, 2, and 3 weeks following phage injections. Phage (1×10^7 TU/well) was coated on microtiter wells (Nunc MaxiSorp; Nunc, Naperville, Ill.) overnight at 4°C. Wells were washed five times with 10 m*M* Tris, 0.15 *M* NaCl, pH 7.5, containing 0.1% (v/v) Tween 20 (TTBS). Wells were then blocked with 1% (w/v) casein in TBS, pH 7.4, (Pierce, Rockford, Ill.) at ambient

temperature for 2 h. After washing with TTBS again, mouse serum (diluted 1:1000), 73 ng rabbit IgG (Sigma I5006), or 73 ng rabbit α-M13 IgG (Sigma B7786) was added followed by incubation for 2 h at ambient temperature. Wells were washed again and goat α-mouse IgG HRP (Sigma 4416) diluted 1:4000 or donkey α-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, Calif.) diluted 1:2000 were added to detect the binding of mouse α-M13 IgG or rabbit α-M13 IgG, respectively. After 2 h at ambient temperature the wells were washed again and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS) was added according to the manufacturer's instructions (Sigma). Color reactions were read at 405 nm using a plate reader (Bio-Tek, Santa Barbara, Calif.; model EL310).

Results

Survival

All mice in this study, except two, lived to the end of the study period. The first mouse died prior to receiving any peptide-phage injections while in the restraint used to hold the mouse for injections. The second mouse died under anesthesia during the second tumor surgery. This appeared to be due to excess administration of halothane anesthesia. The mouse up to that time showed normal behavior and appearance.

Weight

Mice in group 2 which had been injected with φAmp1 × showed an average decrease in body weight of 9.1% on day 1 following injection but their weights had returned to baseline by day 2. Mice in group 4 showed a minor weight loss the day after surgery but this had returned to normal the following day. The weights of all other mice remained stable relative to the control mice.

Activity, behavior and appearance

During the first day after surgery, group-4 mice were less active but had returned to normal by the next day. All other mice had normal activity, behavior and appearance throughout the study.

Histopathologic evaluation

Histologic analyses of 320 organs from 32 mice injected with phage were performed. In group 2, three FVB mice had hepatic inflammation and one FVB mouse had lymphoid aggregates in the hepatic lobules. Sections of liver from mice with hepatic inflammation were subsequently evaluated using a silver stain (Steiner) to rule out *Helicobacter* or *Clostridium* infection. Although these tests were negative, they do not test for all causes of infectious hepatitis in mice. All other organs in all other mice appeared normal for that strain.

IHC detection of phage particles

In group-1 mice, 3 days after injection most tissues were negative. In this group, the spleens from all phage-injected mice (n=4) showed trace to 1+ staining in the germinal centers of the lymphocytes. At 3 weeks after phage injection all organs were negative. In group-2 mice, all tissues were negative for phage staining 3 days after injection of φAmp1 ×. Most tissues from mice injected with φAmp2 × were negative at 3 days with the exception of three livers, two spleens, one lymph node, and one kidney. In group-2 mice all tissues 3 weeks after phage injection were negative except for the liver of one mouse and a lymph node of another mouse, both from the φAmp2 ×-injected group. In group-3 mice, all tissues were negative for phage 3 weeks after phage injection. Among the group-4 mice, the organs of one mouse were available for end-experiment IHC detection of phage and all tissues were negative.

Titering of phage from organs and blood

In group-1 mice, all organs were negative at 3 weeks in the phage-titering assay. In group-2 mice, 3 days after φAmp1 × phage injection, there were infective phage present in all the tissues except the blood and liver of mouse no. 3 and the spleen of mouse no. 2. In this group, no phage were detected in any of the tissues collected 3 weeks after injection of either φAmp1 × or φAmp2 ×. In group-3 mice, blood was free of infective phage 11 days after the third and final injection of peptide-phage. No infective phage were detected in any of the tissues collected 3 weeks after the third injection of phage. Among the group-4 mice, one mouse was available for 3-week titering and most tissues were positive.

Endotoxin test results

Initial RPL preparations contained roughly 10^5 times more endotoxin than is permissible for intravenous administration to humans. Using the Triton X-114 extractions (see Methods section), the amount of endotoxins decreased by several orders of magnitude to levels permissible by the FDA for administration to humans.

Immune response

In response to phage injection, serum IgG levels increased. Serum levels had increased by the end of the 1st week over baseline and continued to increase following serial injections (Fig. 1).

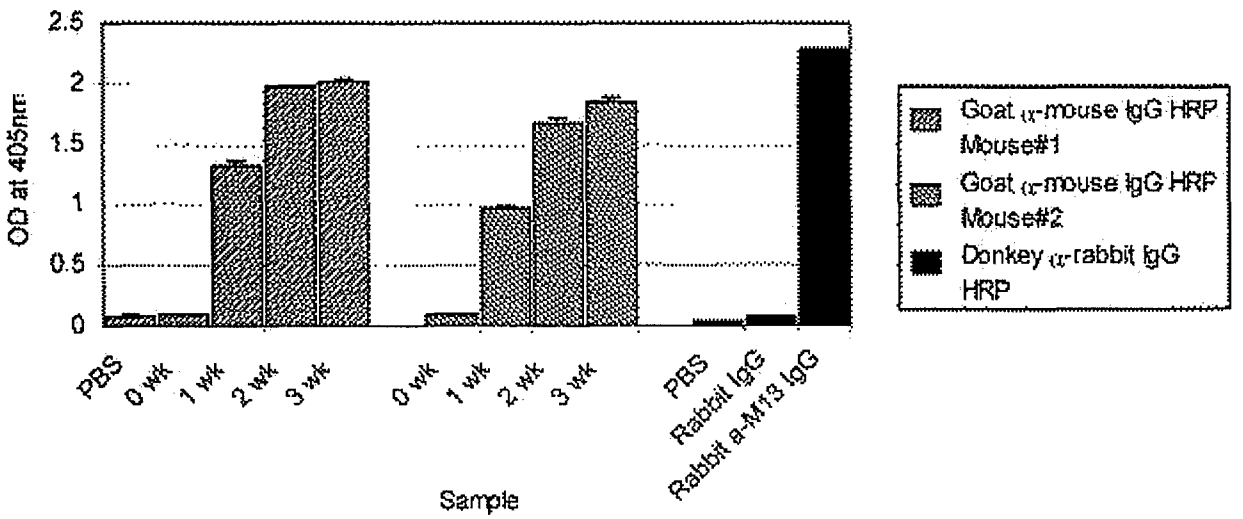

Fig. 1. Mouse serum IgG following i.v. administration of library phage, analyzed by ELISA. Mouse no. 1 was given two doses of phage over 3 days. Mouse no. 2 was given three doses over 6 days

Consensus amino acid sequences of phage recovered from tumors of MRL mice

Among the group-4 mice, 20-70 clones per mouse were sequenced and several amino acid sequence consensus patterns emerged (Fig. 2). Of particular interest, one consensus pattern had strong homology with a peptide previously shown to bind to and inhibit matrix metalloproteinases (MMPs) 2 and 9 (consensus C in Fig. 2). These molecules are strongly associated with the metastatic phenotype and are promising tumor targets [21].

Consensus A:	Clone:
C G S A Y R S P G A C	IV092499-01
C G S A Y R S P G A C	IV092499-02
C G F M S A V P G P C	IV092499-03
C G A F R F L V K D C	IV092499-04
C G D A L P L V N F C	IV092499-05
C D S G G L P L A S C	IV092499-06
C D S G G L P L A S C	IV092499-69
C S Y L P D R S R F C	IV092499-07
C S Y L P D R S R F C	IV092499-56
C S Y L P D R S R F C	IV092499-64
C V S Y S M P P A L C	IV020200-03
C V S Y S M P P A L C	IV020400-54
C G M V S M S P L S C	IV092499-08
C Y H M V S L E N G C	IV092499-09
C V M T S F P W M R C	IV092499-10
Consensus B:	
C E N F V G R N V E C	IV092499-11
C E N F V G R N V E C	IV092499-12
C N M L S L S I P G C	IV092499-39
C N M K V W A T G K C	IV092499-14
C R D L V W R P Q A C	IV092499-13
C R D L V W R P Q A C	IV092499-42
Consensus C:	
C S L W R H W P Y I C	IV080599-16
C W R H W V S N Y D C	IV092499-15
C T G H W G I G E N C	IV092499-16
C T T H W G F T L C	Koivunen[1]
C S L H W G F W W C	Koivunen
C R R H W G F E F C	Koivunen
Consensus D:	
C S H P S M S R G S C	IV020400-14
C S I S E M S R G A C	IV020400-44

[1] Sequences previously reported by panning RPL to purified MMPs.[21]

Fig. 2. Consensus amino acid sequences of peptide-phage isolated from tumor tissue. Amino acids that appear at least two times in vertical alignments are *underlined and in bold type*. Amino acids that are similar but not identical are *underlined and not in bold type*. Although the end cysteines were constant in all peptides and homology may not be as significant as the amino acids within the loop, they are still underlined when they line up in the consensus sequence to emphasize which peptides fall into an identical register with respect to the disulfide loop

Discussion

Bacteriophages have been injected intravenously into thousands of humans and even neonates for the diagnosis and evaluation of immune function and disorders. Although they are very uncommon, non-fatal adverse reactions in patients with unusual genetic immune deficiencies have been reported over the past 30 years [8, 16, 34, 39, 55]. These injections have been performed singly or serially, similar to the process of in vivo screening performed in our mouse study. There has also been extensive use of over 250 strains of bacteriophage, including 39 that infect *Escherichia* bacteria, which have been administered orally, externally, or intraperitoneally for treatment of infection [25, 52, 56]. Not only have side effects been reported as extremely rare, but the phage treatments have been reported to be effective in eliminating the bacterial infection.

Consistent with the reported literature on administration of phages to humans, our study in mice demonstrated that injection of naive and amplified phage using a variety of dose schedules resulted in minimal toxicity. One mouse died prior to receiving any phage injections and one mouse died intraoperatively during the second surgical procedure. The second mouse was well and acting normally until the second surgery. It appeared that the mouse had an excessive exposure to halothane that was irreversible. No other cause of death was discernible.

Out of 32 mice, 4 had identifiable hepatic abnormalities. Only the FVB mice had these findings. Histologic findings included focal acute lobular hepatitis with no evidence of fibrosis. Focal processes were diffusely distributed in small foci. Each nidus consisted of apoptotic and degenerating hepatocytes primarily associated with neutrophils in the larger foci. These histologic findings were present in two of the four mice. A third had mild changes with no necrotic hepatocytes and the fourth mouse had lymphoid aggregates only. No other mice, including those that underwent multiple phage injections had any hepatic changes. While it is possible that this strain had underlying hepatitis or are particularly sensitive to phage, future in vivo studies will be designed to take into account the possibility of hepatic reactions to phage injection.

Acute anaphylactic allergic reactions were a main concern and none was observed despite repeated injections of phage. Another concern was exposure to toxic peptides following injection of enriched phage populations. Following enrichment of phage there are higher copy numbers of enriched peptide sequences. However, the final number of enriched peptide molecules would be extremely small ($\leq$20 pg) and the lack of expected toxicity was supported by the study results.

Previous studies on panning in a murine model have utilized single injections per mouse [2, 36, 41]. Mice were killed and immersed in liquid nitrogen, and the phage collected and amplified, and injected into a next mouse. Such a panning scheme is not possible in humans with cancers. Our mouse study was designed to mimic planned human studies so as to allow serial panning in cancer patients. The fact that minimal toxicity was noted with single and repeated injections suggests that repeat panning in humans is feasible.

Clearance of phage was evaluated by IHC and titering. IHC appeared to be less sensitive than titering for detecting phage in organs. Most organs were IHC-negative for phage within 3 days. Virtually all were negative by 3 weeks. Titering demonstrated phage at 3 days in most tissues in most mice. Blood was negative by 11 days and all tissues in most mice by 3 weeks. An exception was one group-4 mouse that had positive titers in all organs at 3 weeks. These findings are similar to biodistribution of a phage library displaying a Fab fragment evaluated in nude mice [61].

Elevated IgG levels were found 1 week following the last serial injection of peptide-phage library. The levels increased during the 2nd and 3rd week. This response is normal and documents the immune competency of the mice used in this study. This is important since side effects related to immunologic reactions are potentially the most serious in humans. These findings support the validity of these experiments as a relevant preclinical model for subsequent human studies.

It was very encouraging that consensus amino acid sequence patterns were identified from peptide-phage eluted from tumor tissue, and especially, that a peptide was identified that has strong homology to an inhibitor of MMPs associated with the metastatic phenotype [21]. Interestingly, the lymph node tumors in these mice, while multiple and large, do not appear to metastasize or invade tissue outside the capsule of the lymph node. The tumor tissue histologically appears to be similar to normal lymph node tissue. However, for tumors to grow this large (more than a centimeter), a substantial amount of tissue remodeling must take place, and MMPs are likely to play a prominent role in this process.

There are several potential benefits of panning human cancers in vivo rather than on purified material ex vivo or on whole cells. Panning in a human exposes the peptide-phage library to exclusively human targets as opposed to a human xenograft model. A tumor is composed of a complex of tumor cells and host stroma such as fibroblasts and blood vessels. A human xenograft is not purely human but contains host murine blood vessels, stroma, and blood components. Vascular targets in a xenograft model are not human and may be sufficiently different to not be clinically applicable. Human tumors are spontaneous as opposed to the artificial setting of a xenograft model in which host tumor relationships may be quite different. An additional potential advantage of in vivo panning is that all tumor targets will be in their native conformation with all their human post-translational modifications.

An additional benefit of in vivo panning is that phage that have cross-reactive affinity to nonmalignant targets are negatively selected against tumor tissue. This makes cross-reactive phage less available to the tumor. This may be a powerful feature of this method since it should minimize cross-reactivity to nonmalignant targets. The importance of maximizing specificity through subtraction is highlighted by the unexpected cardiac cytotoxicity of Herceptin which appears to be related to cross-reactive binding to the heart [*50*].

Unlike in vitro panning in which a known purified target is panned against, target identification is not necessary for initial in vivo panning events. For targeted therapy with ligand/toxin conjugates, it may be only necessary to know that the target is unique to the cancer. In addition, in vivo panning also allows selection of stable peptides since only peptides that are durable in blood will be able to bind.

We conclude that serial administration of a naive and enriched phage-displayed RPL is minimally toxic in a murine model. The majority of phage appeared to be cleared by 3 days and with few exceptions were nondetectable at 3 weeks by IHC and phage titering. An immune response occurred but, at least under the study conditions, anaphylactic reactions were not observed. In vivo RPL screenings performed serially in the same animal resulted in enrichment of peptide-phage. Several amino acid sequence motifs were identified and one was highly homologous to a known MMP ligand. The results from this study have led to approval by the FDA to begin human cancer patient studies at the University of Vermont similar to the preclinical mouse study presented here.

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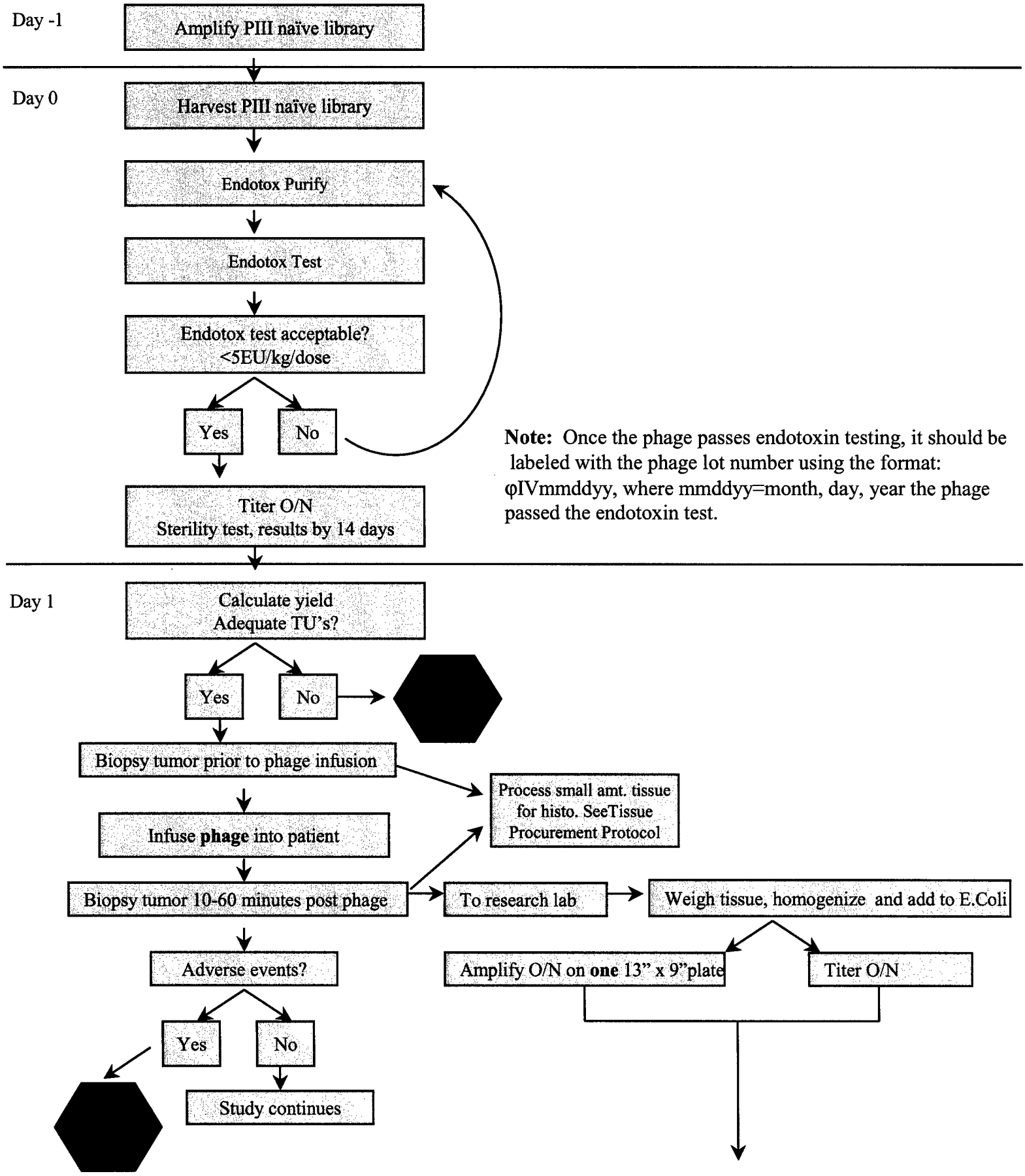
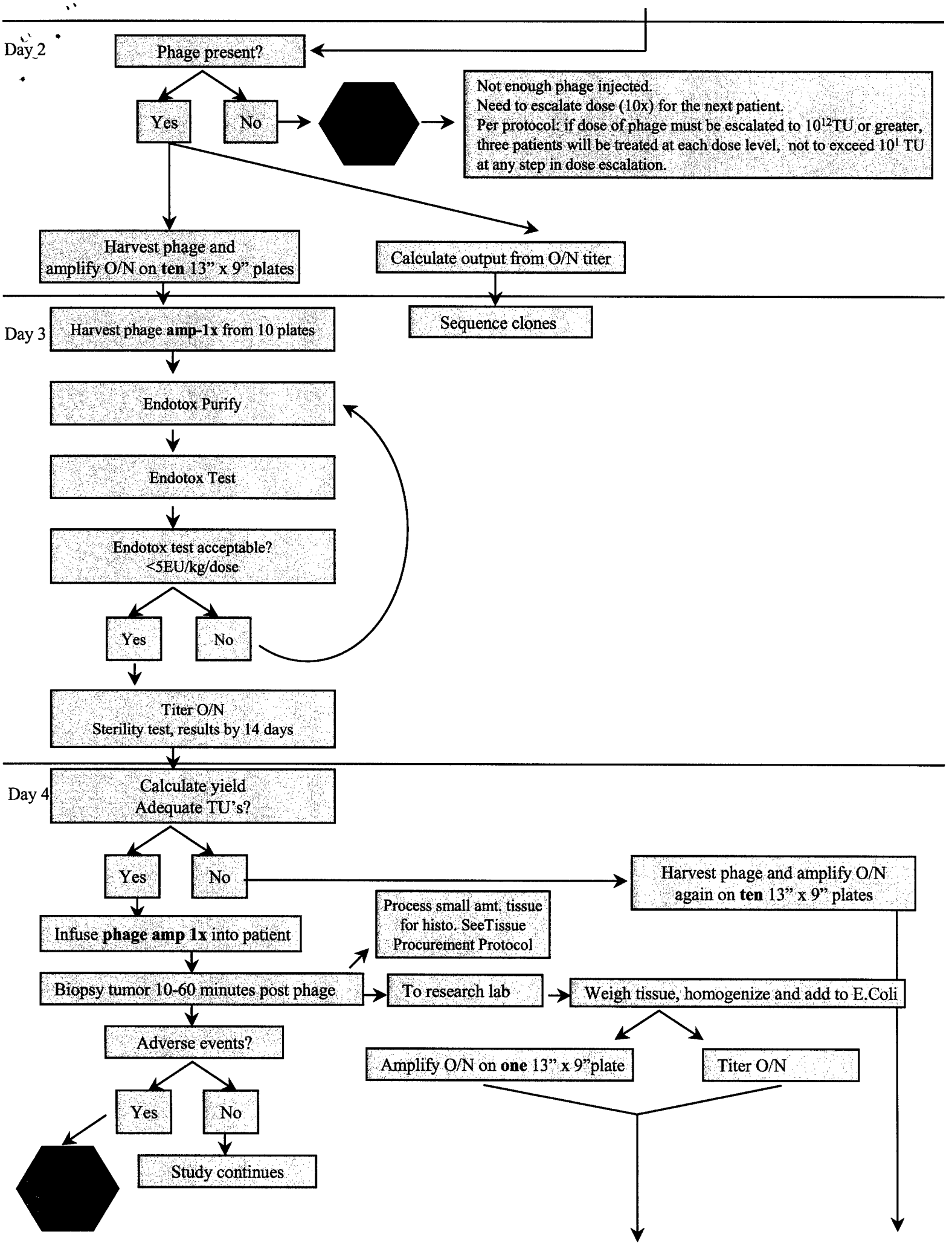
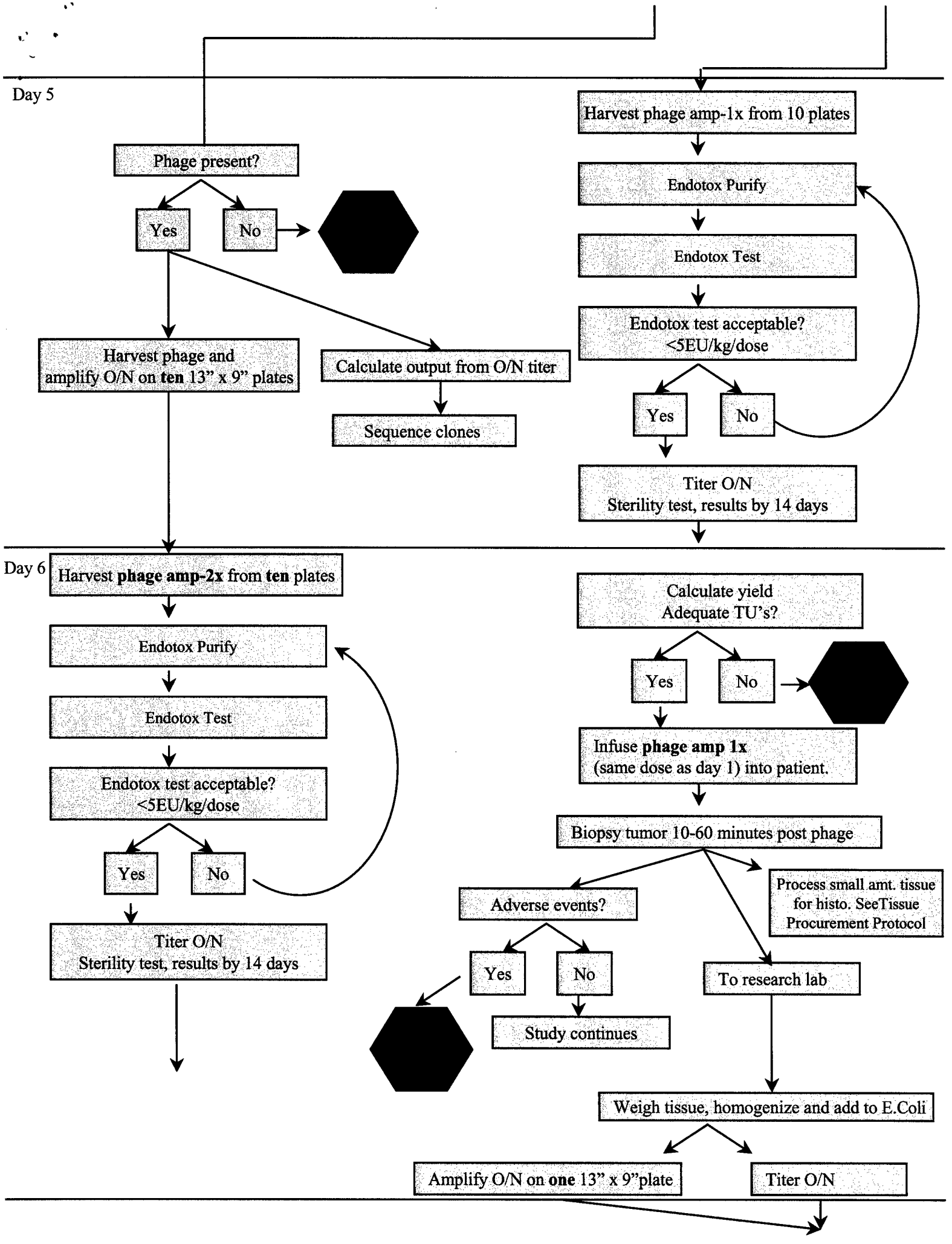
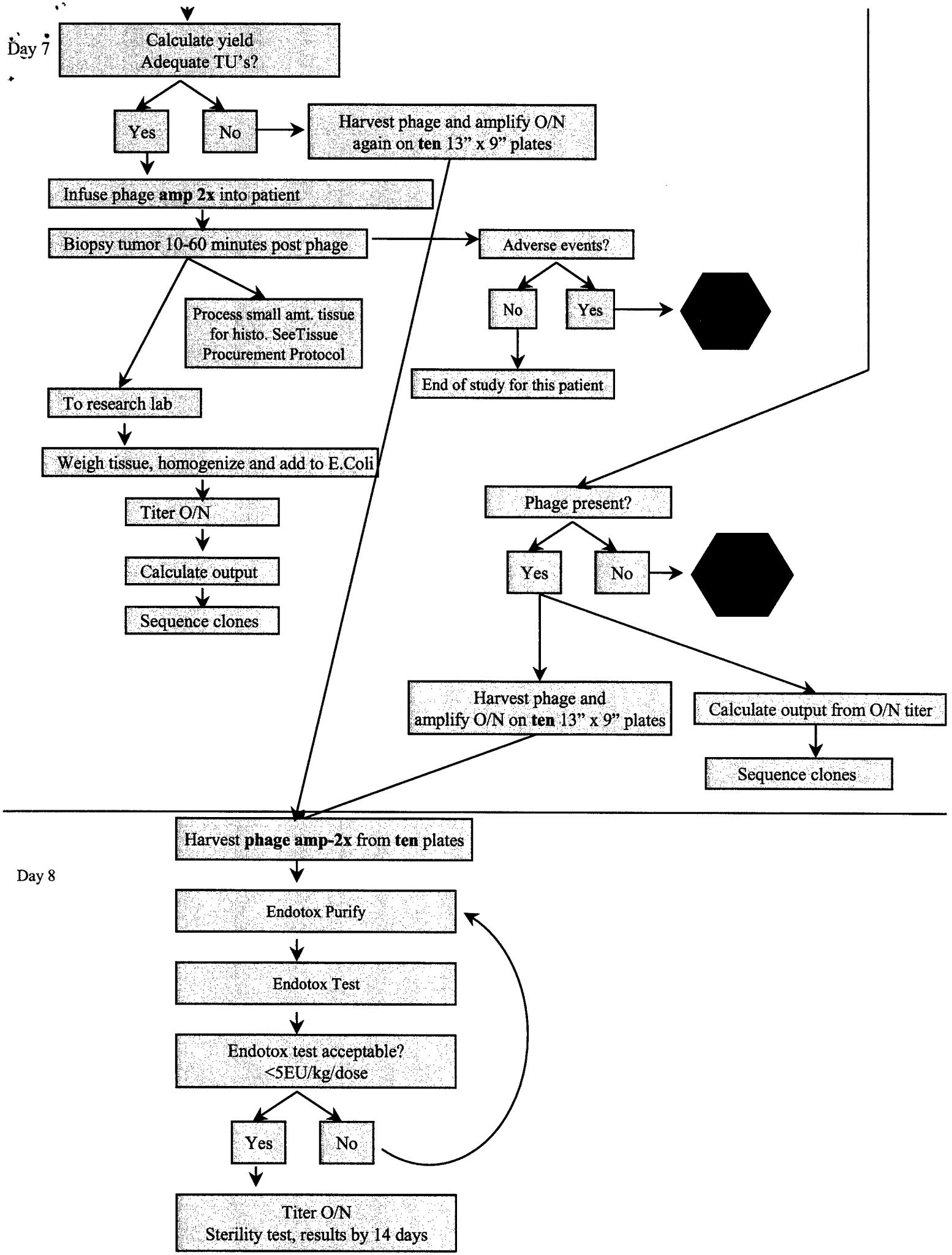
In Vivo Selection of Ligands for Targeted Therapy: Research Laboratory Flow Chart for Peptide Phage



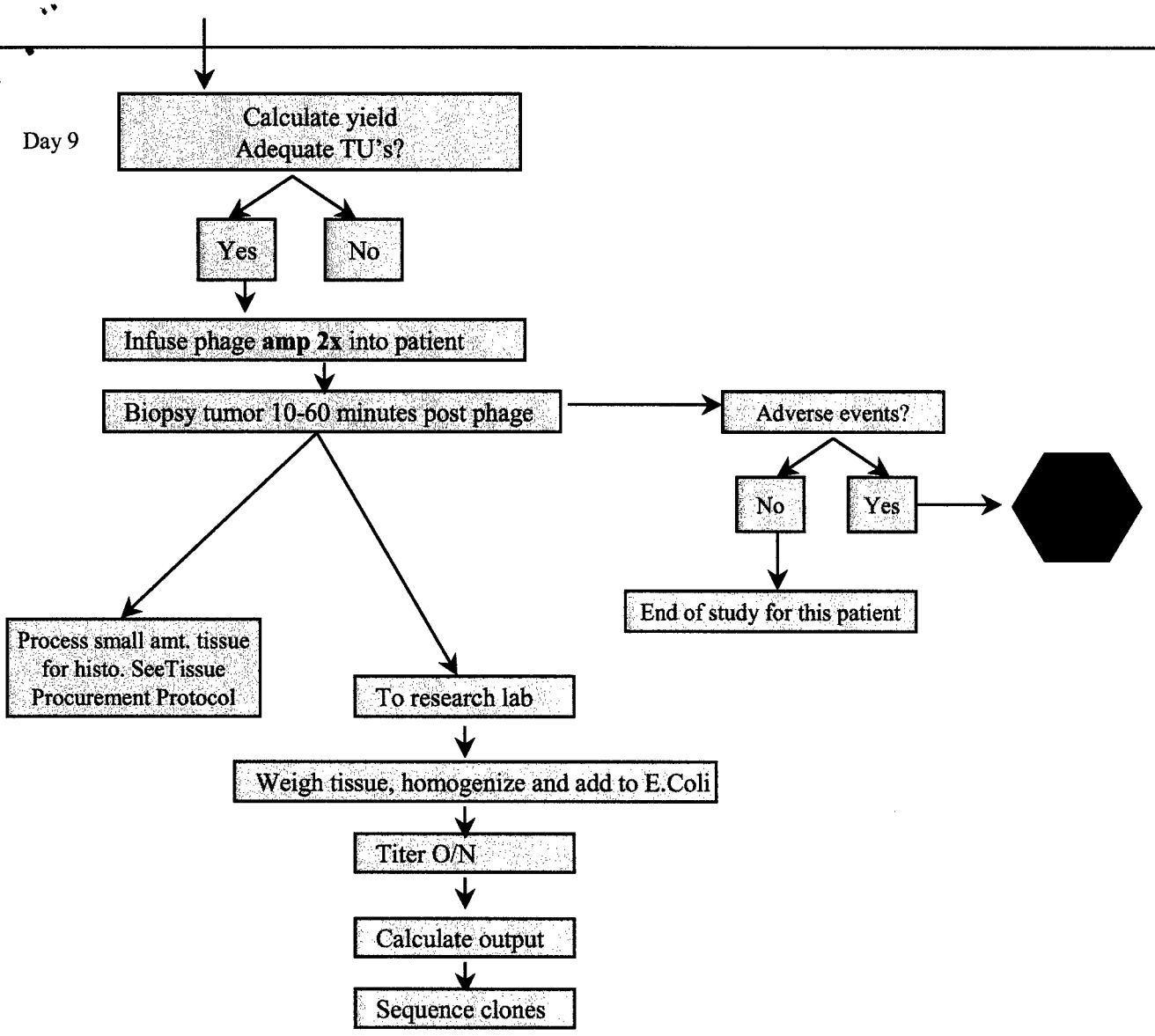
Note: Once the phage passes endotoxin testing, it should be labeled with the phage lot number using the format: φIVmmddyy, where mmddyy=month, day, year the phage passed the endotoxin test.


Day 2
Phage present?
Yes
No
Not enough phage injected.
Need to escalate dose (10x) for the next patient.
Per protocol: if dose of phage must be escalated to 10^{12}TU or greater, three patients will be treated at each dose level, not to exceed 10^{1} TU at any step in dose escalation.
Harvest phage and amplify O/N on **ten** 13" x 9" plates
Calculate output from O/N titer
Day 3
Harvest phage **amp-1x** from 10 plates
Sequence clones
Endotox Purify
Endotox Test
Endotox test acceptable? <5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Day 4
Calculate yield
Adequate TU's?
Yes
No
Harvest phage and amplify O/N again on **ten** 13" x 9" plates
Infuse **phage amp 1x** into patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Biopsy tumor 10-60 minutes post phage
To research lab
Weigh tissue, homogenize and add to E.Coli
Adverse events?
Amplify O/N on **one** 13" x 9"plate
Titer O/N
Yes
No
Study continues

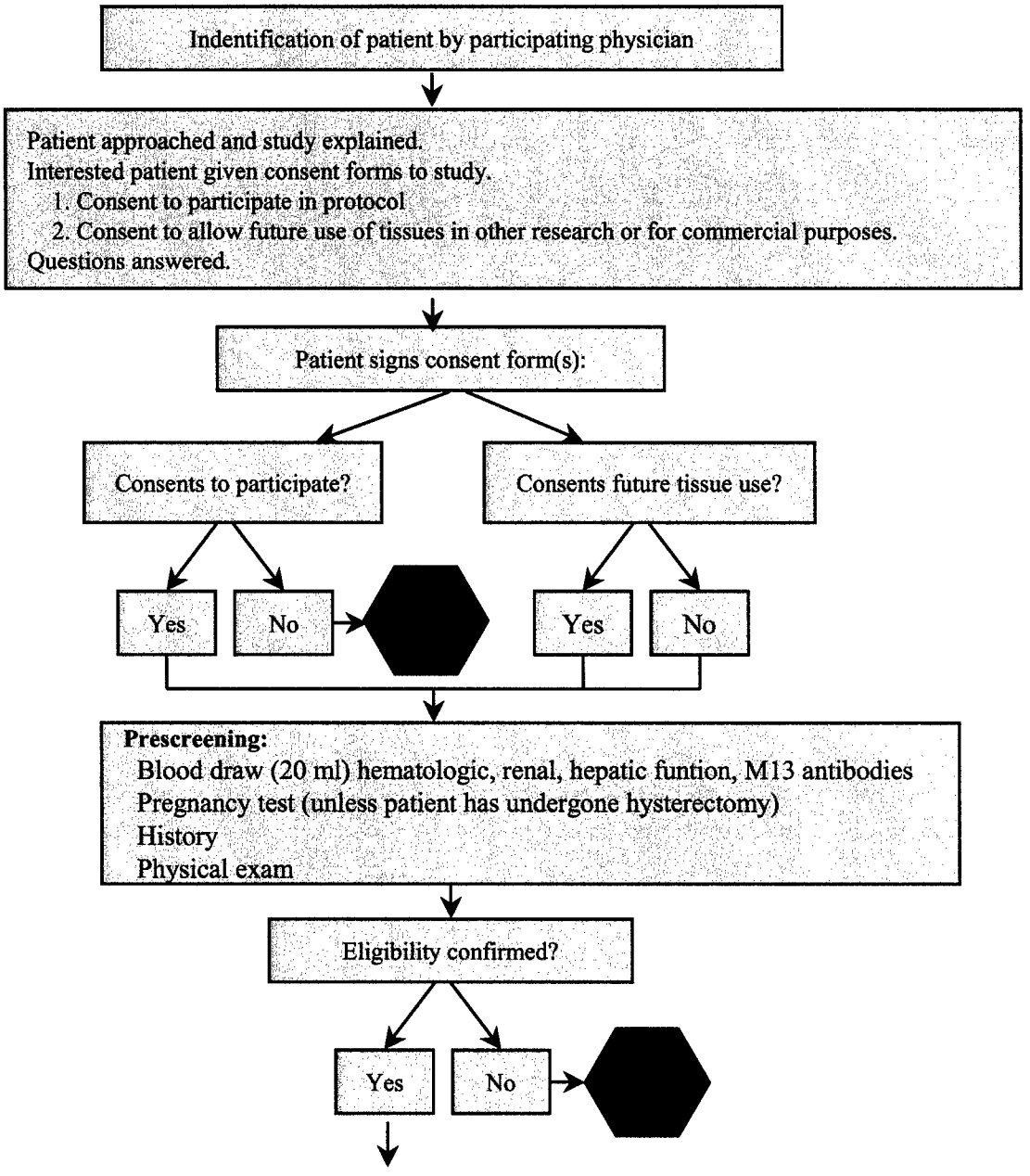

Day 5
Harvest phage amp-1x from 10 plates
Endotox Purify
Endotox Test
Endotox test acceptable?
<5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Phage present?
Yes
No
Harvest phage and
amplify O/N on ten 13" x 9" plates
Calculate output from O/N titer
Sequence clones
Day 6
Harvest phage amp-2x from ten plates
Endotox Purify
Endotox Test
Endotox test acceptable?
<5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days
Calculate yield
Adequate TU's?
Yes
No
Infuse phage amp 1x
(same dose as day 1) into patient.
Biopsy tumor 10-60 minutes post phage
Adverse events?
Yes
No
Study continues
Process small amt. tissue
for histo. SeeTissue
Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Amplify O/N on one 13" x 9"plate
Titer O/N


Day 7
Calculate yield
Adequate TU's?
Yes
No
Harvest phage and amplify O/N again on **ten** 13" x 9" plates
Infuse phage **amp 2x** into patient
Biopsy tumor 10-60 minutes post phage
Adverse events?
No
Yes
End of study for this patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Titer O/N
Calculate output
Sequence clones
Phage present?
Yes
No
Harvest phage and amplify O/N on **ten** 13" x 9" plates
Calculate output from O/N titer
Sequence clones
Harvest **phage amp-2x** from **ten** plates
Day 8
Endotox Purify
Endotox Test
Endotox test acceptable?
<5EU/kg/dose
Yes
No
Titer O/N
Sterility test, results by 14 days

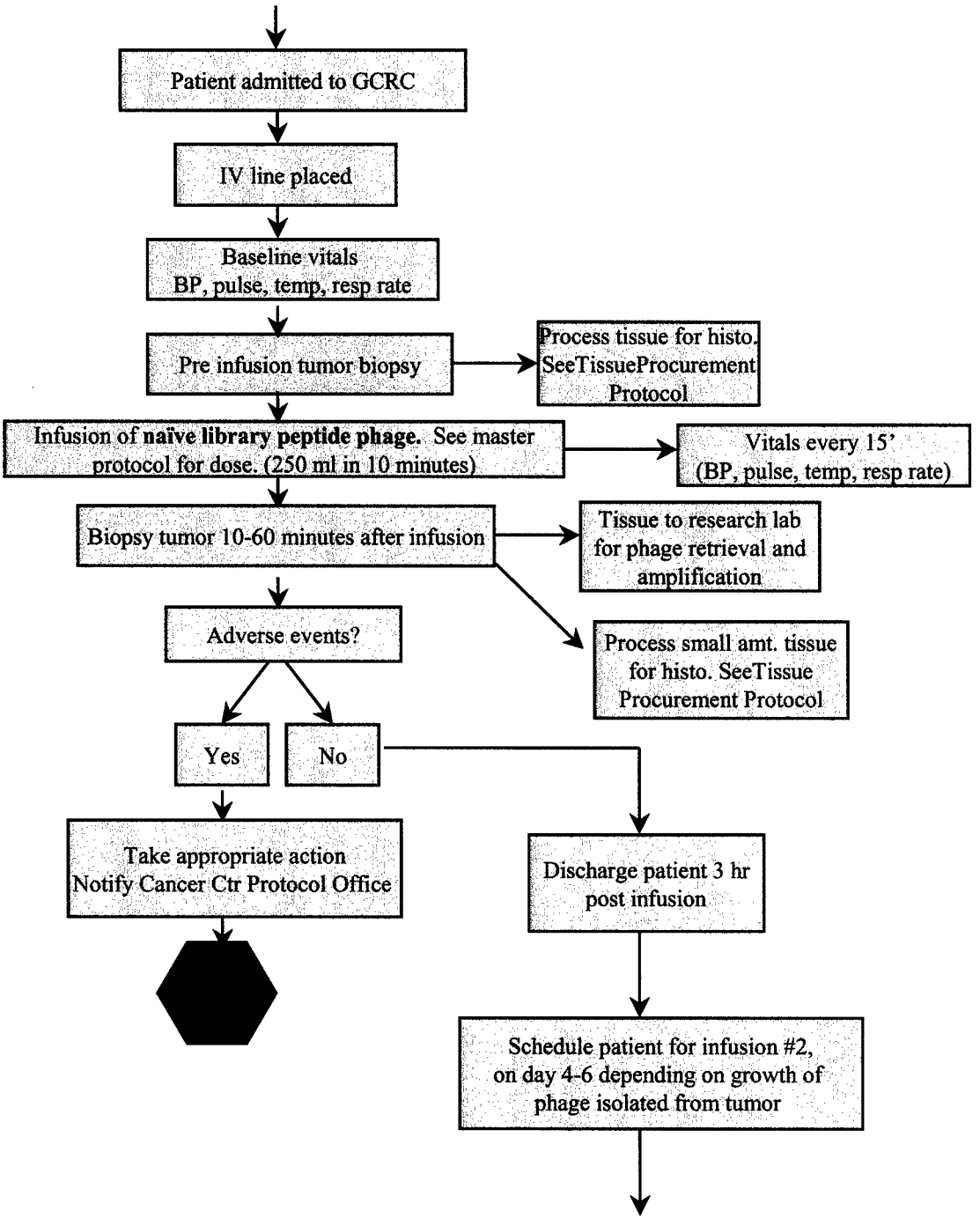
Day 9


Calculate yield
Adequate TU's?
Yes
No
Infuse phage **amp 2x** into patient
Biopsy tumor 10-60 minutes post phage
Adverse events?
No
Yes
End of study for this patient
Process small amt. tissue for histo. SeeTissue Procurement Protocol
To research lab
Weigh tissue, homogenize and add to E.Coli
Titer O/N
Calculate output
Sequence clones

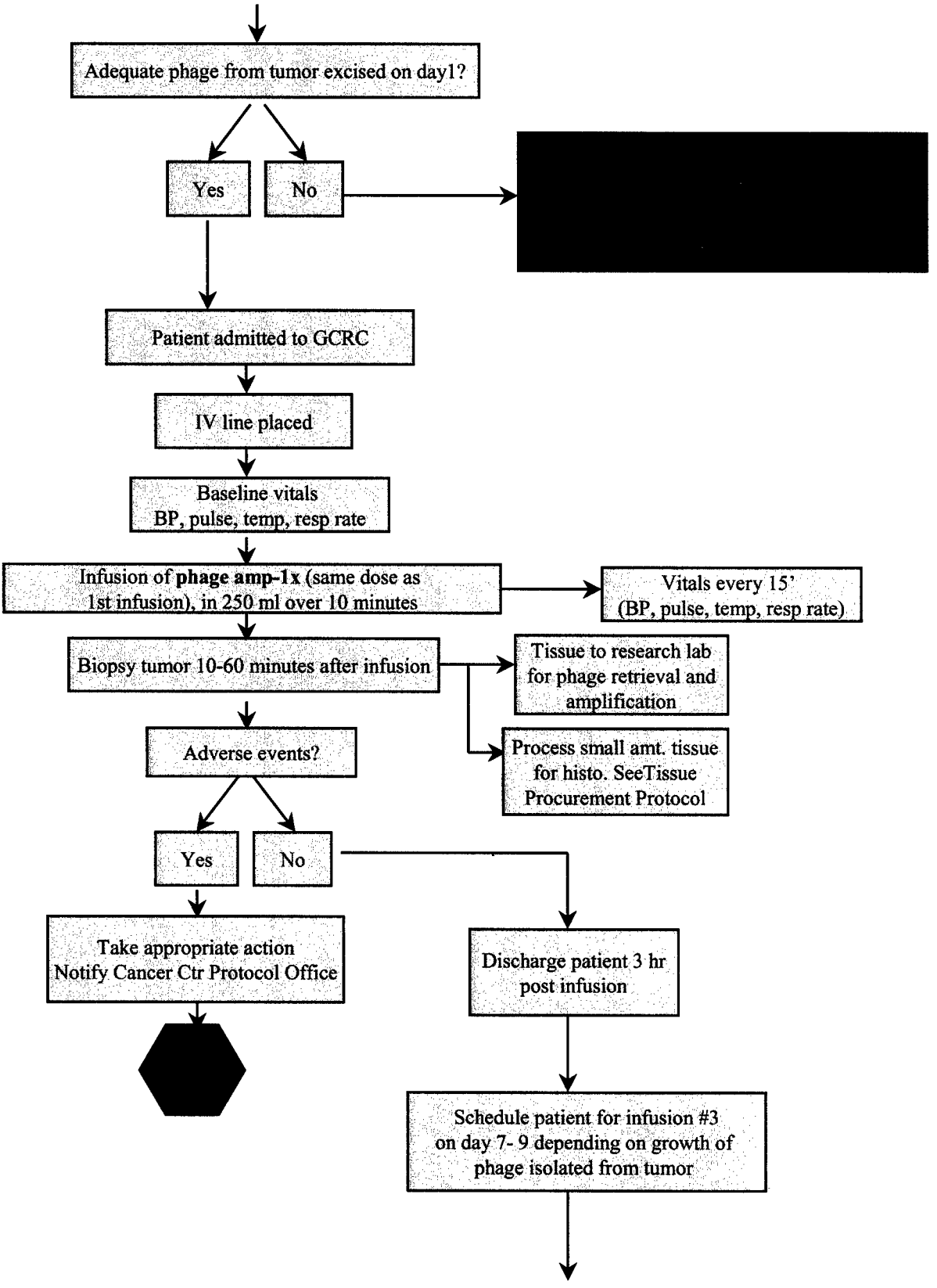
In Vivo Selection of Ligands for Targeted Therapy: Patient Flow Chart



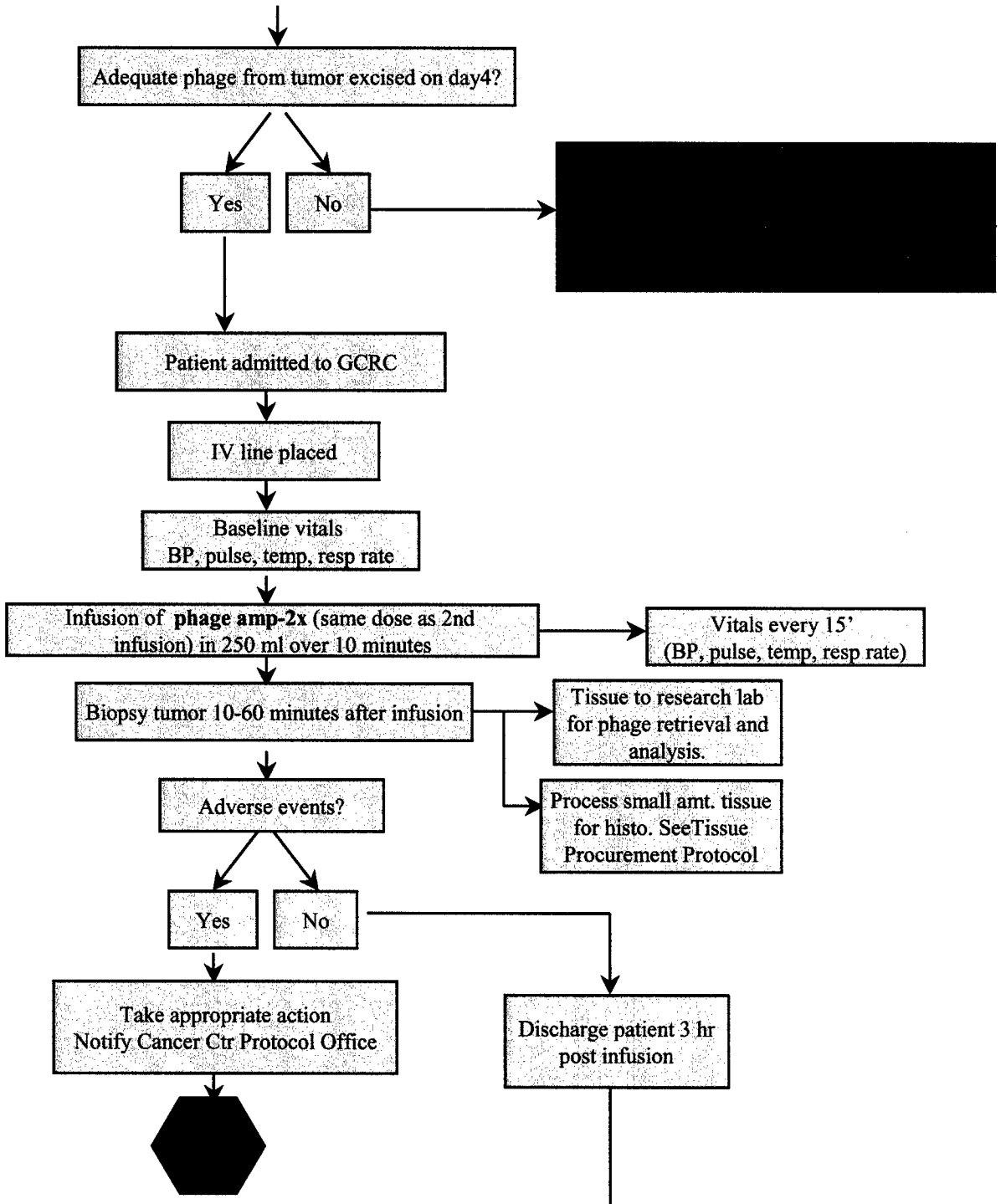
Day 1


Patient admitted to GCRC
IV line placed
Baseline vitals
BP, pulse, temp, resp rate
Pre infusion tumor biopsy
Process tissue for histo. SeeTissueProcurement Protocol
Infusion of naïve library peptide phage. See master protocol for dose. (250 ml in 10 minutes)
Vitals every 15'
(BP, pulse, temp, resp rate)
Biopsy tumor 10-60 minutes after infusion
Tissue to research lab for phage retrieval and amplification
Adverse events?
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Yes
No
Take appropriate action
Notify Cancer Ctr Protocol Office
Discharge patient 3 hr post infusion
Schedule patient for infusion #2, on day 4-6 depending on growth of phage isolated from tumor

Day 4
or
Day 6


Adequate phage from tumor excised on day1?
Yes
No
[redacted]
Patient admitted to GCRC
IV line placed
Baseline vitals
BP, pulse, temp, resp rate
Infusion of **phage amp-1x** (same dose as 1st infusion), in 250 ml over 10 minutes
Vitals every 15'
(BP, pulse, temp, resp rate)
Biopsy tumor 10-60 minutes after infusion
Tissue to research lab for phage retrieval and amplification
Process small amt. tissue for histo. SeeTissue Procurement Protocol
Adverse events?
Yes
No
Take appropriate action
Notify Cancer Ctr Protocol Office
Discharge patient 3 hr post infusion
Schedule patient for infusion #3 on day 7- 9 depending on growth of phage isolated from tumor

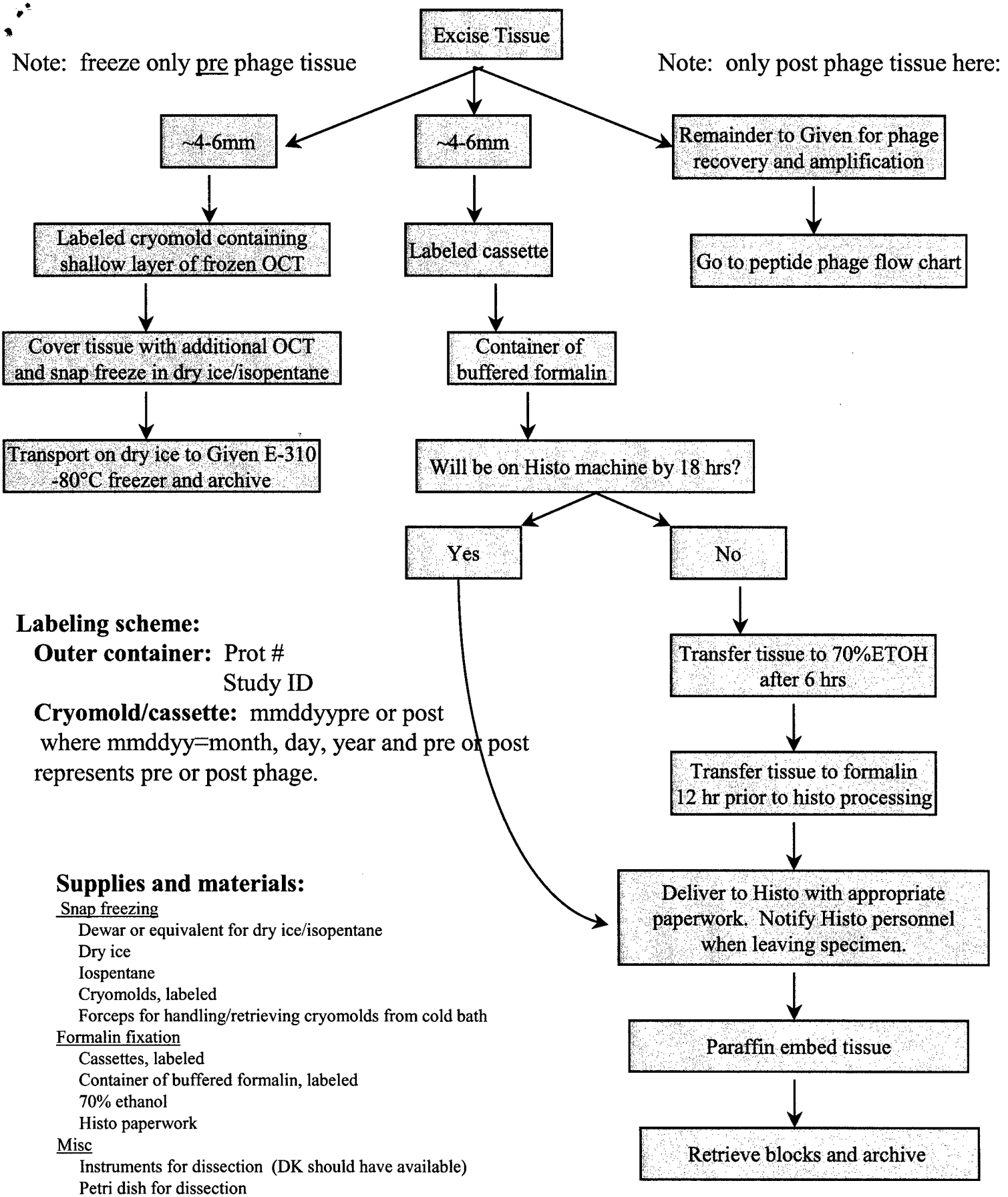
Day 7
or
Day 9



One month after last phage infusion

Six weeks to 2 months after last phage infusion

Tissue Procurement Flow Chart



Labeling scheme:

Outer container: Prot #
Study ID

Cryomold/cassette: mmddyypre or post
where mmddyy=month, day, year and pre or post represents pre or post phage.

Supplies and materials:

Snap freezing
- Dewar or equivalent for dry ice/isopentane
- Dry ice
- Iospentane
- Cryomolds, labeled
- Forceps for handling/retrieving cryomolds from cold bath

Formalin fixation
- Cassettes, labeled
- Container of buffered formalin, labeled
- 70% ethanol
- Histo paperwork

Misc
- Instruments for dissection (DK should have available)
- Petri dish for dissection
- Gloves

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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